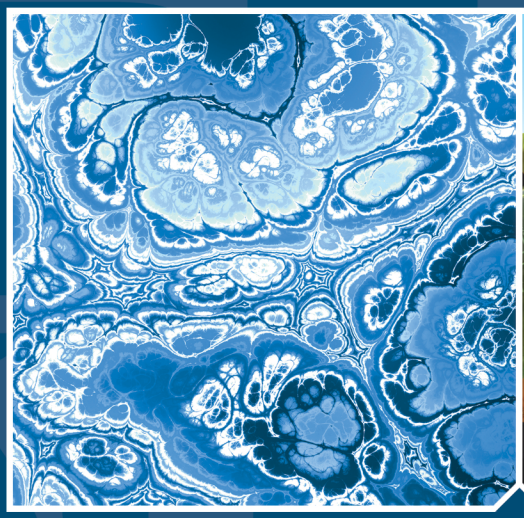
United States
Environmental Protection
Agency

Proceedings of the 2009 U.S. Environmental Protection Agency Workshop on Innovative Approaches for Detecting Microorganisms and Cyanotoxins in Water

MAY 20 - 21, 2009
REGION 3 OFFICES
PHILADELPHIA, PA



Office of Research and Development
National Center for Environmental Research

The 2009 U.S. Environmental Protection Agency Workshop on Innovative Approaches for Detecting Microorganisms and Cyanotoxins in Water

May 20 - 21, 2009

EPA Region 3 Offices
Shenandoah Room, #104
1650 Arch Street
Philadelphia, PA

Workshop Objectives

- Provide a forum to discuss proposed solutions to the methodological challenges in the search for better methods of detection and assessment of waterborne microbial contaminants.
- Facilitate collaboration and cooperation among scientists and policy-makers from research entities, EPA, states, local agencies, and stakeholders.
- Assist EPA in identifying what research or technologies are needed to better inform decisions and/or policies associated with the assessment of microorganisms in water.
- Give STAR grantees of the past two solicitations regarding "Development and Evaluation of Innovative Approaches for the Quantitative Assessment of Pathogens and Cyanobacteria and Their Toxins in Drinking Water" the opportunity to present their latest findings. Summaries of the grantees' projects can be found at: http://epa.gov/ncer/rfa/2005/2005_pathogens_drinking_water.html and http://cfpub.epa.gov/ncer_abstracts/index.cfm/fuseaction/recipients.display/rfa_id/456/records_per_page/ALL

Wednesday, May 20, 2009

1:00 p.m. **Welcome and Overview of EPA's Office of Research and Development and the Science To Achieve Results (STAR) Program**
Barbara Klieforth, EPA, Office of Research and Development, National Center for Environmental Research

1:25 p.m. **OGWDW Microbial Research Needs from a Regulatory Perspective**
Sandhya Parshionikar, Team Leader, Microbiology Technical Support Center
Office of Ground Water and Drinking Water

1:55 p.m. **Overview Presentation From EPA Region 3**
Victoria P. Binetti, EPA, Region 3

2:15 p.m. **Crypto and Molecular Methods Work Being Done With EPA Regions 2 and 3**
Eric Villegas, EPA, National Exposure Research Laboratory, Microbiological and Chemical Exposure Assessment Research Division

Wednesday, May 20, 2009 (continued)

2:35 p.m.	**Development of a Universal Microbial Collector (UMC) for Enteric Pathogens in Water and Its Application for the Detection of Contaminant Candidate List Organisms in Water** Kelly R. Bright, University of Arizona
2:55 p.m.	**Break**
3:15 p.m.	**Development and Evaluation of an Innovative System for the Concentration and Quantitative Detection of CCL Pathogens in Drinking Water** Saul Tzipori, Tufts University
3:35 p.m.	**On-Chip PCR, Nanoparticles, and Virulence/Marker Genes for Simultaneous Detection of 20 Waterborne Pathogens** Syed Hashsham, Michigan State University
3:55 p.m.	**Rapid and Quantitative Detection of *Helicobacter pylori* and *Escherichia coli* O157 in Well Water Using a Nano-Wired Biosensor and QPCR** Evangelyn C. Alocilja, Michigan State University
4:15 p.m.	**Assessment of Microbial Pathogens in Drinking Water Using Molecular Methods Coupled With Solid-Phase Cytometry** Barry Pyle, Montana State University
4:35 p.m.	**Detecting Pathogens in Water by Ultrafiltration and Microarray Analysis** Anthea K. Lee, Metro Water District of Southern California
5:00 p.m.	**Adjourn**

Thursday, May 21, 2009

8:30 – 9:00 a.m.	**Robust Piezoelectric-Excited Millimeter-Sized Cantilever Sensors for Detecting Pathogens in Drinking Water at 1 Cell/Liter** Raj Mutharasan, Drexel University
9:00 – 9:20 a.m.	**National Risk Management Research Laboratory (NRMRL) Microbial Research** Jorge Santo Domingo, EPA, NRMRL, Water Supply and Water Resources Division, Microbial Contaminants Control Branch
9:20 – 9:40 a.m.	**Rapid Concentration, Detection, and Quantification of Pathogens in Drinking Water** Zhiqiang Hu, University of Missouri

Thursday, May 21, 2009, (continued)

9:40 – 10:10 a.m.	**Simultaneous Concentration and Real-Time Detection of Multiple Classes of Microbial Pathogens From Drinking Water** Mark D. Sobsey, University of North Carolina at Chapel Hill
10:10 – 10:30 a.m.	**Break**
10:30 – 10:50 a.m.	**Quantitative Assessment of Pathogens in Drinking Water** Kellogg Schwab, Johns Hopkins University
10:50 – 11:40 a.m.	**Discussion on the Next Generation of Methods and Research Needs**
11:40– noon.	**Development and Application of a Fiber Optic Array System for Detection and Enumeration of Potentially Toxic Cyanobacteria** Donald Anderson, Woods Hole Oceanographic Institute
12:00 – 1:10 p.m.	**Lunch**
1:10 – 1:30 p.m.	**Development of High-Throughput and Real-Time Methods for the Detection of Infective Enteric Viruses** Jason Cantera, University of California at Riverside
1:30 – 1:50 p.m.	**New Electropositive Filter for Concentrating Enterovirus and Norovirus From Large Volumes of Water** Mohammad Karim, Oak Ridge Institute for Science and Education Research Fellow, EPA
1:50 – 2:10 p.m.	**Automated Methods for the Quantification and Infectivity of Human Noroviruses in Water** Timothy Straub, Batelle Pacific Northwest Division
2:10 – 2:30 p.m.	**Characterization of Naturally Occurring Amoeba-Resistant Bacteria From Water Samples** Sharon Berk, Mid-Tennessee State University
2:30 – 2:50 p.m.	**Break**
2:50 – 3:10 p.m.	**Analysis of Various Toxins Produced by Cyanobacteria Using Ultraperformance Liquid Chromatography-Tandem Mass Spectrometry (UPLC/MS/MS)** Stuart Oehrle, Northern Kentucky University
3:10 – 3:20 p.m.	**Development of Sensitive Immunoassay Formats for Algal Toxin Detection** Fernando Rubio, Abraxis LLC
3:20 – 4:00 p.m.	**Wrap-up & Adjournment**

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This workshop was intended to facilitate progress on the quantitative assessment of microbial agents in water and bring research scientists together with policy makers. EPA's success is dependent, in large part, on its ability to make credible environmental decisions based on solid scientific information and technical methodologies. Reliable, sensitive, robust, and versatile detection and monitoring tools are needed to address the risk assessment and management of known and emerging microbial contaminants in source water, treated water, and/or distribution systems. The goal of this workshop was to foster discussion on the development of cost-effective, timely, and innovative technology solutions in assessing and managing environmental risks to human health.

Workshop Objectives

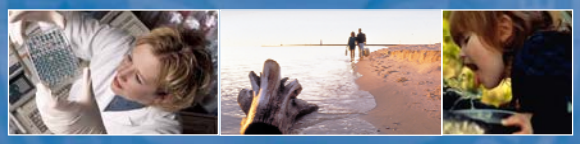
- Provide a forum to discuss proposed solutions to the methodological challenges in the search for better methods of detection and assessment of waterborne microbial contaminants.

- Facilitate collaboration and cooperation among scientists and policy makers from research entities, EPA, states, local agencies, and other stakeholders.

- Assist EPA in identifying what research or technologies are needed to better inform decisions and/or policies associated with the assessment of microorganisms in water.

- Give Science To Achieve Results (STAR) grantees of the past two solicitations regarding "Development and Evaluation of Innovative Approaches for the Quantitative Assessment of Pathogens and Cyanobacteria and Their Toxins in Drinking Water" an opportunity to present their latest findings. Abstracts of the grantees' projects can be found at: http://epa.gov/ncer/rfa/2005/2005_pathogens_drinking_water.html and http://cfpub.epa.gov/ncer_abstracts/index.cfm/fuseaction/recipients.display/rfa_id/456/records_per_page/ALL

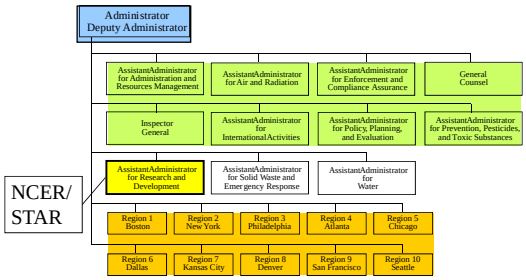
The STAR Drinking Water Program



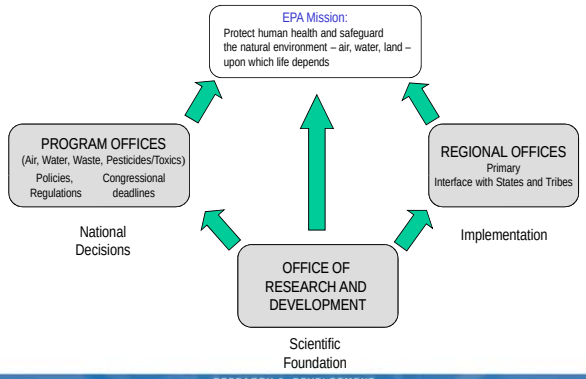
Barbara Klieforth,
National Center for Environmental Research

EPA Organization

Mission: To protect public health and to safeguard and improve the natural environment - air, water, and land - upon which life depends



Support for EPA's Mission



NCER's Extramural Programs

Science To Achieve Results (STAR)

- ***Targeted Research Grants through RFAs***
- ***Exploratory/Futures Grants***
- ***Graduate Fellowships***
- ***Competed Centers***

Greater Research Opportunities

Earmarks

Small Business Innovation Research (SBIR) Contracts

Experimental Program to Stimulate Competitive Research (EPSCoR)

Grantees and fellows are among the top scientists in the country

EPA STAR Program

- **Established in 1995 as part of the overall reorganization of ORD**
- **Mission: include this country's universities and nonprofit groups in EPA's research program and ensure the best possible quality of science in areas of highest risk and greatest importance to the Agency**
- **Issue approximately 20-25 RFAs each year**
- **Each year: receive 2500-3200 grant applications**
- **Award about 250-300 new STAR grants, fellowships & SBIR contracts per year**
- **Manage about 1000 active research grants and fellowships**

EPA STAR Research Program

- **Goal-directed solicitation planning**
- **Significant cross-agency and interagency involvement with solicitation planning, writing, and review**
- **Competitive solicitations**
- **Joint Solicitations with other Agencies**
- **External peer review**
- **Internal relevancy review:** program office and regional input
- **Fund highest priority projects**
  - Grantees and fellows are among the top scientists in the country
- **Communicate research results through website, ORD laboratories, program office and regional meetings, and publications *(www.epa.gov/ncer)***

STAR Results in Action: Regulations and Voluntary Actions

- Results from the Marshfield Clinic Research Foundation's research led to major, statewide changes (e.g., UV disinfection) in treatment of water from groundwater sources *(Borchardt)*
- UNC at Chapel Hill examined impacts of water distribution systems in contributing to GI illness, results are included in considerations for updating the Total Coliform Rule (*Tolbert*)
- Results used by EPA's Office of Water in preparing: "Economic, Environmental, and Benefits Analysis of the proposed Metal Products & Machinery Rule" *(Herriges)*
- University of Iowa findings on mechanisms and kinetics of chloramine loss & byproduct formation in distribution systems used in the Stage 2 Disinfectants and Disinfection Byproducts (DBP) rule published in 2006
- STAR research results on "integrated pest management" used by cities & states to reduce childrens' exposures to pest allergens
- STAR research findings led to voluntary industry action – protective clothing and hand-washing facilities for agricultural workers expected to reduce "take home" pesticide exposures

STAR Results in Action:

Tools and Methods for Decision Making

- University of Maryland's Center for Marine Biotechnology's 1st of its kind PCR technique that rapidly detects *Helicobacter pylori* in environmental samples. *H. pylori* had previously been extremely difficult to detect because of its ability to transform into a non-culturable form.
- STAR researchers developed molecular detection techniques for pfisteria – used by states and CDC for real time monitoring of pfisteria events
- STAR research developed promising method for assessing pesticide concentrations in saliva – accurate & less invasive method to quantify exposure & dose
- Rapid assessment protocol for stream biomass developed – used in OW guidance document and by states
- Research played a key role in the preparation of a manual on economic valuation for the British Department of Environment, Regions, and Transport *(Carson)*

STAR & SBIR Results in Action:

Practical Applications

- Tufts' U. alternative method (portable continuous flow centrifuge) for concentrating low numbers protozoa from large volumes of water approved as an alternative concentration method by EPA (Tzipori)
- Soybean oil plastics being used to manufacture tractor parts for John Deere (Wool)
- Developed a benign catalyst to replace chlorine in oxidation processes (Collins)
- Developed a substitute for lead solder now used broadly in the electronics industry (Wong)
- STAR-supported grant research has led to new, environmentally friendly packaging manufactured by Cargill-Natureworks and used by the Wal-Mart Corporation Advanced Technology Materials, Inc developed dry scrubber using deposition for semiconductor industry. Business grew from five partners to 1100 employees and sales over $250 million (NASDAQ : ATMI)

STAR Results in Action:

Education

- **New course in green engineering**
- **Fellows are now professors in many, major universities**
- **Fellows are working in government agencies**
- **Fellows elected to 36 scientific panels and/or advisory committees**
- **Sustainability curricula expanded in many universities as a direct result of P3**
- **Four new small businesses created because of P3**

Science To Achieve Results (STAR) Program

NCER's Drinking Water Program

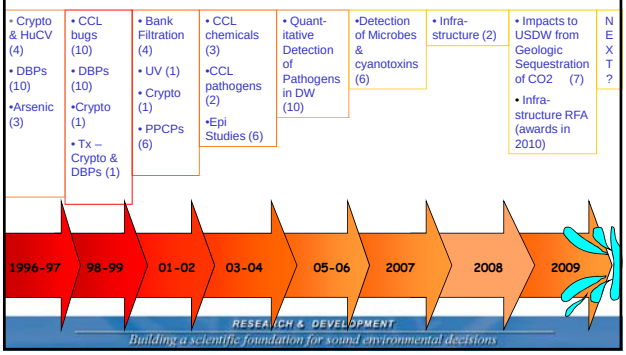
- **Program begun in FY 1996**
- **Funding levels historically between $2.5-5.0 M/yr**
- **NCER has been funded research in a wide variety of areas**
- **Research completed 3-4 years after award**
- **Solicitation preparation and Programmatic Reviews have extensive participation from OW, ORD, and Regional Offices**

Drinking Water (& Water Quality)

- Current components
  - Identifying and quantifying microbes in water
  - Decision making for water infrastructure sustainability
  - Source water/aquifer protection from potential impacts of geologic sequestration of carbon dioxide
- Recent solicitations
  - *Integrated Design, Modeling, and Monitoring of Geologic Sequestration of Anthropogenic Carbon Dioxide to Safeguard Sources of Drinking Water*
  - *Development and Evaluation of Innovative Approaches for the Quantitative Assessment of Pathogens and Cyanobacteria and Their Toxins in Drinking Water*
  - *Innovative and Integrative Approaches for Advancing Public Health Protection Through Water Infrastructure Sustainability*

History of STAR Drinking Water Projects

http://epa.gov/ncer/science/drinkingwater/recipients.html



RESEARCH & DEVELOPMENT
Building a scientific foundation for sound environmental decisions

Science To Achieve Results (STAR) Program

Other Water-related RfAs

Some examples:

- Forecasting Ecosystem Services from Wetland Condition Analyses **(2008)**
- Enhancing Ecosystem Services from Agricultural Lands **(2009)**
- Watershed Classification **(2002, 2003)**
- Ecological Thresholds **(2004)**
- EcoHABs

RESEARCH & DEVELOPMENT
Building a scientific foundation for sound environmental decisions

OGWDW Microbial Research Needs from a Regulatory Perspective

The U.S. Environmental Protection Agency Workshop on Innovative Approaches for Detecting Microorganisms and Cyanotoxins in Water
May 20-21, 2009

Sandhya Parshionikar, PhD
Technical Support Center
Office of Ground Water and Drinking Water
U.S. Environmental Protection Agency

Overview

- The SDWA requirements and regulatory process.
- Research input in Drinking water regulations
- Sources of data used
- Research Needs
  - General
  - Specific issue
  - Total Coliform Rule
    - Revisions
    - Research and Information Collection Partnership
  - Long term

Safe Drinking Water Act

- SDWA requires regulation of contaminants that:

- May have an adverse health effect
  - must consider sensitive sub-populations of infants, children, pregnant women, elderly, individuals with history of serious illness
- Occur or are likely to occur in PWSs (considering frequency and level)
- Present a meaningful opportunity for health risk reduction
  - based on best available science and data

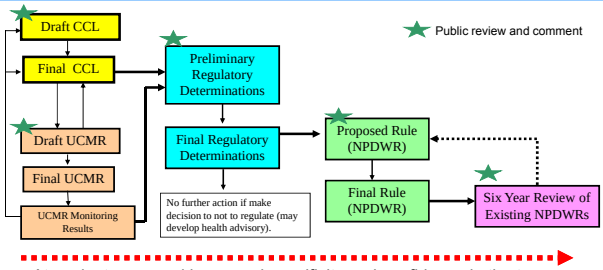
Safe Drinking Water Act Requirements

- EPA must publish Maximum Contaminant Level Goals (MCLGs)
  - Must set levels at which no health effects occur and which allows for adequate margin of safety
  - Required EPA to regulate specific microbial contaminants (viruses, Giardia, Legionella, total coliforms, heterotrophic bacteria)
- EPA must promulgate MCLs or treatment technique requirement as close to the MCLG as is "feasible" (taking costs into consideration)
  - Required EPA to set treatment technique requirements for surface and ground water systems to protect for pathogens

Safe Drinking Water Act Requirements

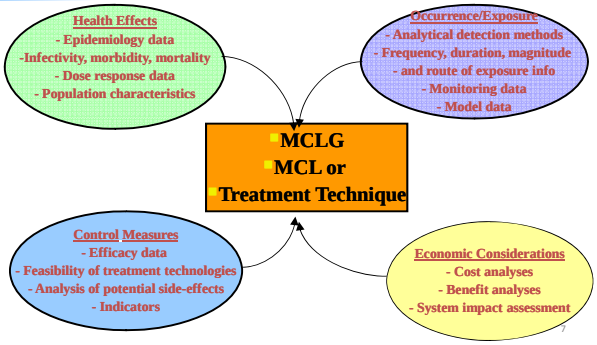
- EPA must develop Contaminant Candidate List (CCL) for unregulated contaminants every 5 years
  - Establish criteria for a program (UCMR) to monitor unregulated contaminants, and to identify no more than 30 contaminants to be monitored, every five years.
  - Perform regulatory determination on five of CCL contaminants every five years
- Requiring the Agency to review and revise, as appropriate, each National Primary Drinking Water Regulation no less often than every 6 years
  - Revisions must assure public health protection (the net effect of the rule must be to maintain or improve public health protection)

Generalized Flow of Regulatory Processes



At each stage, need increased specificity and confidence in the type of supporting data used (e.g. health, occurrence and treatment).

Research Inputs into DW Regulations



Sources of Research Data Used

- EPA Office of Research and Development
  - In house research
  - STAR grants
- Regions
- Water Research Foundation (formerly AwwaRF)
- Contracts with Universities and research institutions
- Interagency agreements
- Co-operative agreements
- Other published, peer reviewed literature

Regulatory Drivers: Some Near Term Examples

- CCL 4
- UCMR 4
- Regulatory Determinations 3
- DS information collection
- 6 year review

Research Needs: General

- Exposure Data
  - Analytical Methods
    - Innovative approaches to measurement
    - Practical implementable technologies
  - Occurrence data
    - Outbreak analyses
    - Endemic prevalence
  - Epidemiological studies
- Health effects
  - Dose response
  - Subpopulations affected
  - Host factors involved

Research Needs: General

- Treatment
  - Behavior of pathogens under different types of treatment conditions
  - Novel strategies for contamination mitigation
- Other research
  - Pathogen virulence
  - Role of host factors in infectivity
  - Fate and transport of pathogens under environmental conditions

Research Needs: Examples of Specific Issues

- Methods that detect pathogen infectivity/viability/strain identification
- Exposure to pathogens from drinking water contamination events
- Role of Biofilms in pathogen exposure and their impact on chlorine residuals
- Survival of nucleic acids under various treatments
- Innovative approaches for sampling and detection
- Research in Support of Revised TCR/DS

Total Coliform Rule (TCR)

2000 - Stage 2 Federal Advisory Committee (FAC) Agreement In Principle (AIP) suggested review of distribution system issues with the 6-year review of the TCR

2003 - Six year review of existing drinking water regulations → ***TCR should be revised***

2007 - Federal Advisory Committee convened to provide recommendations on

- how EPA should revise the TCR, and
- what research and information collection should be conducted to better inform distribution system risk

Total Coliform Rule Revisions

The Advisory Committee developed an AIP to be the foundation for the proposed rule

- A more proactive approach to public health protection
- Use of monitoring results shift from informing public notification to informing investigation and corrective action

  2010: Propose rule revisions

  2012: Final rule

  2015: compliance starts
- Includes recommendations for distribution system research and information collection and the formation of a Research and Information Collection Partnership

Research and Information Collection Partnership (RICP)

Recommended by TCR Federal Advisory Committee to:

- Inform and support the drinking water community to develop future risk management decisions regarding drinking water distribution systems
- Partnership formed January 29, 2009 between EPA and Water Research Foundation
- Steering Committee provides input on research and information collection priorities
  - 3 members from EPA
  - 3 members from water utilities
  - 3 additional members
  - Public health
  - Environmental
  - State Regulator

Research and Information Collection Partnership (RICP)

- Develop a research agenda to identify decision relevant research and information collection needs or priorities

  - Biofilms
  - Nitrification
  - Intrusion
  - Storage
  - Contaminant Accumulation
  - Main Repair
  - Cross Connection Control

  -First Draft Research Agenda – September 2009

  -Initial priorities for research and information collection identified - 2010

Long Term Research Needs

- Online monitoring/Rapid results
  - Perturbations in water quality
  - Outbreak analysis
    - Quantitative
    - Genotyping/Strain identification
    - Sensitivity
- High through put detection
- Universal detection of all classes of pathogens
- Miniaturization of technology
  - Use in field
- Genomics/Proteomics

New STAR RFA

- EPA seeking new and innovative research applications that link opportunities to advance public health protection with improvements in the condition and function of the water infrastructure.
- The focus on improving the effectiveness of the water infrastructure for protecting public health.
- Should clearly demonstrate an integrated, multi-disciplinary approach that leads to advances in design, operation, and management of the water infrastructure and should directly tie those advances to public health protection in conjunction with improving water efficiency and reducing energy requirements.
- http://www.epa.gov/ncer/rfa/2009/2009_star_water_infrastructure.html.

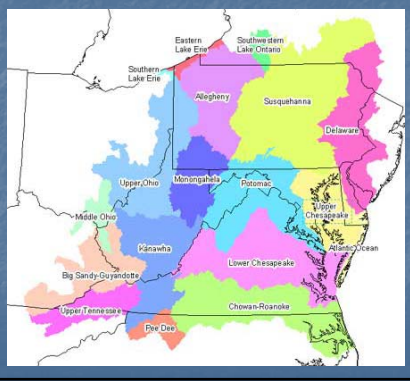
Region 3 Overview

Victoria P. Binetti
US Environmental
Protection Agency

Workshop on Innovative Approaches
For Detecting Microorganisms
And Cyanotoxins in Water

Philadelphia, PA
May 20, 2009

US EPA Mid-Atlantic Region

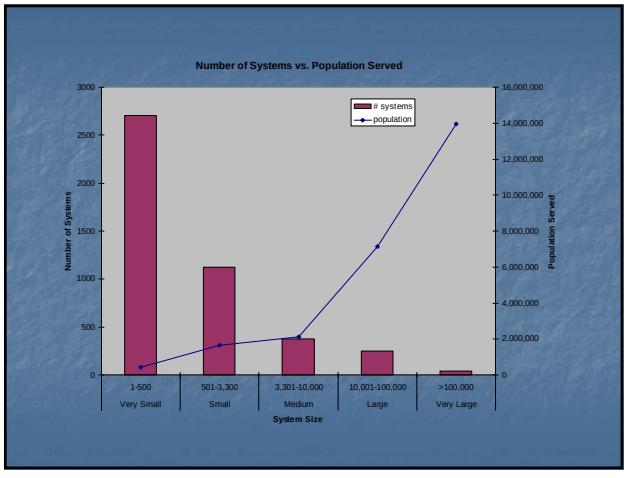


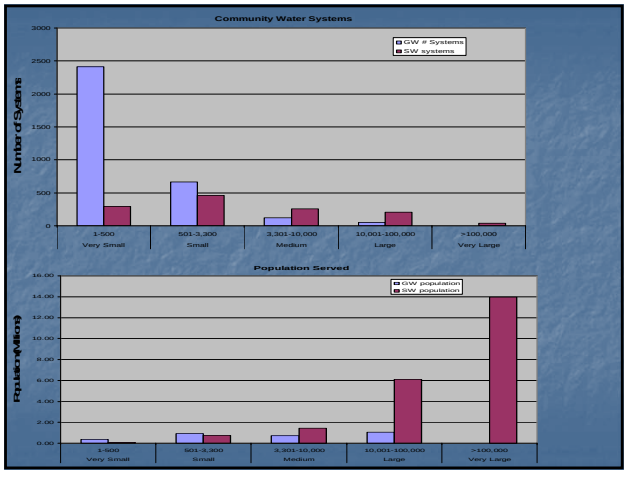
We are employing a "Healthy Waters" strategy to restore and protect our waters by

- Protecting four water uses
  - Aquatic life
  - Recreation
  - Fish consumption
  - Drinking water health
- Reducing causes of impairment
  - Nutrients
  - Sediments
  - Toxics
  - Pathogens

....and by

- By addressing contaminant sources
  - Agriculture
  - Developed/Developing lands
  - Mining
  - Transportation
- Using approaches like
  - Wholesale solutions
  - Prevention partnerships
  - Integrated strategies
  - Green solutions





Some observations on drinking water program implementation in Region 3

- Many public water systems are small, under-resourced, and have limited technical capacity
- Greatest number of violations overall are related to monitoring
- Most frequent health-based violations relate to pathogen regulations: Total Coliform Rule, Surface Water Treatment Rules
- Newer regulations requiring source water sampling are challenging

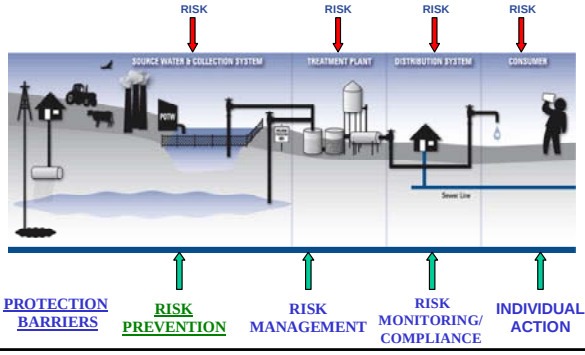
Implementing a multi-barrier approach to safe drinking water:

- Prevent/Reduce pathogens in source waters
- Eliminate/Inactivate pathogens through treatment
- Assess/Monitor to detect pathogen occurrence in finished water
- Assess exposure, health effects

Comprehensive Source Water Protection

MULTIPLE RISKS REQUIRE MULTIPLE BARRIERS

SDWA PROTECTING AMERICA'S PUBLIC HEALTH



Needs today from the field include:

- Monitoring & quantification methods – *Cryptosporidium*, bacteria, viruses
  - Low-cost, reliable
- Tools for viability assessment, speciation
- Pathogen indicators
- Real-time *E. coli* identification
- Efficacy of best management practices for nutrient & sediment control, in prevention of pathogen contamination
- Efficacy of best management practices used for protection of surface waters, in protection of ground water

Issues for the Research Agenda

- Distribution system is the next frontier
- Aging, deteriorating infrastructure increases pathogen exposure risk
- Longer-lived, healthier—but more vulnerable---population?
- Impacts of population growth, climate change and patterns of development on water use and water supply needs will drive treatment and technology – e.g., water efficiency, water reuse, aquifer storage & recovery, etc.
- Climate change will affect pathogen distributions, geographically and seasonally
- Water security concerns will remain—detection, response, recovery

Molecular-Based Detection Systems for *Cryptosporidium* Oocysts

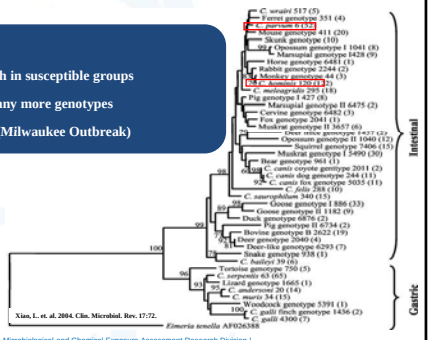
Eric N. Villegas, Ph.D.
STAR Grants Workshop on Innovative Approaches for Detecting Microorganisms and Cyanotoxins in Water
US EPA Region 3, Philadelphia, PA
May 20-21, 2009

Overview

1. Brief introduction to waterborne *Cryptosporidium*
   - Biology and diversity of *Cryptosporidium* species
   - Current detection methodologies
2. US EPA-NERL's waterborne protozoan research program
   - Building a "Protozoan Detection Toolbox"
3. Perspectives on the future of the "Protozoan Detection Toolbox"
   - Future directions and considerations

Cryptosporidium species

- Enteric protozoan parasite
- Chronic diarrhea and death in susceptible groups
- At least 20 species, with many more genotypes
- Waterborne transmission (Milwaukee Outbreak)

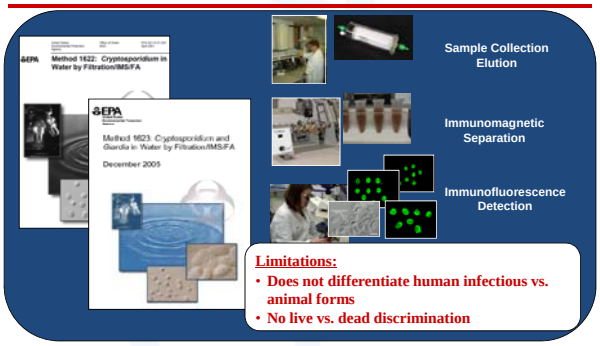


Cryptosporidium Species Infecting Humans and Selected Animals

Host	Major Species	Minor Species
Humans	*C. hominis and C. parvum* (90% of all infections)	*C. meleagridis, C. felis, C. canis, C. suis, cervine genotype*
Cat	*C. felis*	
Cattle	*C. parvum, C. bovis, C. andersoni, deer-like genotype*	*C. suis*
Chickens	*C. baileyi*	*C. meleagridis*
Deer	*C. parvum, deer genotype*	
Dog	*C. canis*	
Turkey	*C. meleagridis, C. baileyi*	
Pig	*C. suis*	*Pig genotype II*
Sheep	*Cervine genotype 1-3, bovine genotypes*	

Modified from Fayer and Xiao. 2008.

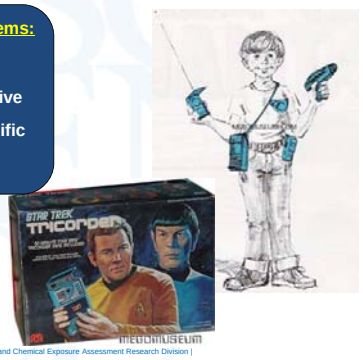
Method 1622/1623: Detection of *Cryptosporidium* and *Giardia*



Limitations:
- Does not differentiate human infectious vs. animal forms
- No live vs. dead discrimination

Challenges for the 21st Century "Water Quality Tricorder"

Protozoan Detection Systems:
1. Fast and user friendly
2. Sensitive and quantitative
3. Species/genotype specific
4. Live vs. dead



Question Driven Research

1. What are the total levels of *Cryptosporidium* in the watershed?
2. How complex is the *Cryptosporidium* species diversity in the watershed?
3. What are the total levels of pathogenic *Cryptosporidium* in the watershed?
4. Are the *Cryptosporidium* oocysts in the watershed viable/infectious?
5. Other questions…

Tracking Sources of Contamination in a Watershed


Cryptosporidium Source Tracking in the Potomac River Watershed

Goals

- Identify types of *Cryptosporidium* oocysts present
- Use PCR-RFLP and Method 1623
- Identify potential sources of *Cryptosporidium* oocysts in the Potomac River

Potential Sources:
Storm water runoffs
Wastewater treatment discharges
Wild animals
Agricultural/animal operations

Methodology

Collection of 20-L water samples (93 samples)

Filtration of two 10-L samples

Method 1623

One filter to an LT2 certified laboratory

Immunomagnetic separation of oocysts

Microscopy

One filter to CDC laboratory

Immunomagnetic separation of oocysts

PCR, DNA sequencing

Species and Genotypes Found

TABLE 5. *Cryptosporidium* genotypes found in water samples in the Potomac watershed

Species or genotype	Major known host(s)	Minor known host(s)	No. of samples positive	No. of detections[a]	Detection site(s)
C. andersoni	Cattle	Sheep, humans (?)	41	167 (151 type A, 14 type B, and 2 type C sequences)	All except Great Seneca Creek[b]
C. felis	Cats	Cattle, humans	2	3	Great Seneca Creek
C. meleagridis	Birds	Humans, dogs, deer mice, brown rats	1	1	Great Seneca Creek
C. serpentis	Snakes, lizards	Squirrels	1	1	Potomac WFP
Deer mouse genotype III (W1)	Deer mice		3	5	Great Seneca Creek, Potomac WFP, Corbalis WTP
Deer mouse genotype IV (W3)	Deer mice		1	1	Great Seneca Creek
Cervine genotype (W4)	Sheep, zoo and wild ruminants, squirrels, chipmunks, woodchucks	Deer mice, beavers, raccoons, lemurs, humans	3	5	Great Seneca Creek
Muskrat genotype I (W7)	Muskrats, voles		3	4	Corbalis WTP, North Fork Shenandoah River, Monocacy River
Snake genotype (W11)	Snakes		1	1	Potomac WFP
W12			1	1	Great Seneca Creek
Skunk genotype (W13)	Skunks	Raccoons, otters, opossums, squirrels, humans	4	5	Great Seneca Creek, Potomac WFP, Corbalis WTP
Vole genotype (W15)	Voles		1	1	North Fork Shenandoah River
Tenrecine genotype	Tenrecs		1	1	Great Seneca Creek
C. hominis-like genotype			1	1	Potomac WFP
Mouse genotype II-like	Mice		1	3	North Fork Shenandoah River

[a] Total number of positive samples for five PCR replicates of all samples.
[b] Detected in one PCR replicate of one storm flow water sample from the Great Seneca Creek.

Yang, et.al. 2008. Applied and Environmental Microbiology

Summary and Impact:

Summary

- A cattle specific species (*C. andersoni)* was the predominant oocyst detected tested
- Pathogenic *C. hominis* and *C. parvum* were not detected in all 93 samples analyzed
- Only minor species/genotypes infecting humans were detected (10 samples)
- Molecular-based detection technique used in this project proves to be sensitive to detect and genotype oocysts in source waters

Impact

- Helped Utilities and Region 3 understand that oocysts in the surrounding county's source water are predominantly non-pathogenic
- Utilities are setting out to work with the agricultural community by encouraging and implementing better management practices (BMPs) in the local cattle/dairy industry

What Lies Ahead for the Waterborne *Cryptosporidium* Research Program?

Multiple Pathogen Detection Systems

Quantitative PCR-Based Detection of *Cryptosporidium spp.*

- Many species and genotypes found in source water
- Most quantitative PCR published have varying degrees of specificities
- Development of multiplex qPCR assays

species	All *Cryptosporidium* spp.	*C. parvum* specific	*C. hominis* specific
C. parvum	+	+	-
C. hominis	+	-	+
C. muris	+	-	-
*C. meleagridis**	+	-	-
*C. felis**	+	-	-
*C. canis**	+	-	-
T. gondii	-	-	-

* Purified genomic DNA from CDC

Molecular Detection Technologies: A Perspective

1. Molecular-based detection of *Cryptosporidium* is in its infancy
2. A better understanding of the differences between zoonotic and human-specific *Cryptosporidium/Giardia* is possible
3. Advances in the "Protozoan Detection Toolbox" will improve our understanding of these parasites and their relationship to public health

Acknowledgements

US EPA
Ann Grimm
Rich Haugland
Michael Ware
Jim Ferretti
Charles Kanetsky
Ron Landy
Marie O'Shea
Kim Roy Young

CDC
Lihua Xiao
Wenli Yang
Vitaliano Cama
Theresa Dearen

Washington Suburban Sanitary Commission
Plato Chen

Frederick County Division of Utilities and Solid Waste Management
Kenneth G. Orndorff

Dynamac, Corp.
Erin Beckman
Reena Mackwan
Abu Sayed

Fairfax Water, Fairfax, VA
Gregory J. Prelewicz

Washington Aqueduct
Miranda H. Brown

Interstate Commission for the Potomac River Basin
Cherie L. Schultz

Questions?

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Development of a Universal Microbial Collector (UMC) for Enteric Pathogens in Water

Kelly R. Bright
Charles P. Gerba
Dept. of Soil, Water & Environmental Science

THE UNIVERSITY OF ARIZONA.

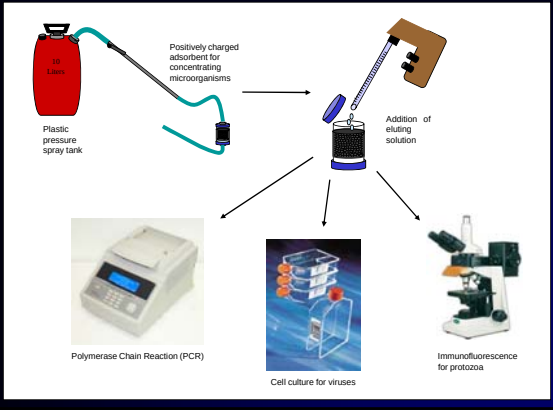
Project Aim

To develop a low cost universal microbial concentrator for application to water.

Universal Microbial Concentrator Requirements

- Simple, easy to operate
- High capacity
- High flow rate
- Low cost
- Concentrates diverse microorganisms
- Elution efficiencies similar to existing methods
- Limit interfering substances

Universal Microbial Concentrator



Identification:

- Culture methods (bacteria)
- Microscopy (parasites)
- PCR/cell culture (viruses)

Cuno Carbon Nanofiber Filters

- Charge-modified granular carbon nanofibers

- Highly porous
- Large surface area
- Highly positively charged

Adsorption of bacteria onto charge-modified carbon nanofibers

Organism	Influent Volume (L)	Influent titer (per liter)	Effluent titer (per liter)	Log_{10} Reduction	Adsorbed microbes (per gram carbon)
Klebsiella terrigena	10	6.7×10^8	< 50	> 7.13	$> 1.1 \times 10^8$
	125	4.4×10^8	< 50	> 6.94	$> 8.8 \times 10^8$
Salmonella typhimurium	10	9.8×10^9	< 50	> 8.29	$> 1.6 \times 10^9$
	125	2.1×10^9	< 50	> 7.62	$> 4.3 \times 10^9$

Adsorption of viruses onto charge-modified carbon nanofibers

Organism	Influent Volume (L)	Influent titer (per liter)	Effluent titer (per liter)	Log_{10} Reduction	Adsorbed microbes (per gram carbon)
MS-2 phage	10	1.9×10^8	< 250	> 5.87	$> 3.0 \times 10^7$
	125	1.2×10^8	< 250	> 5.66	$> 2.3 \times 10^8$
p22 phage	10	3.5×10^8	< 250	> 6.14	$> 5.6 \times 10^7$
	125	1.0×10^8	< 250	> 5.60	$> 2.0 \times 10^8$
fr phage	10	2.5×10^8	< 250	> 5.99	$> 4.0 \times 10^7$
	125	1.4×10^8	< 250	> 5.74	$> 2.8 \times 10^8$
ϕX-174 phage	10	3.4×10^7	< 250	> 5.12	$> 5.4 \times 10^6$
	125	5.5×10^7	< 250	> 5.34	$> 1.1 \times 10^8$
Qβ phage	10	4.0×10^8	< 250	> 6.20	$> 6.5 \times 10^7$
Poliovirus / Rotavirus	10	2.0×10^7	< 111	> 5.26	$> 3.2 \times 10^6$
Adenovirus 40	10	1.0×10^7	< 111	> 4.96	$> 1.6 \times 10^6$
Feline Calicivirus	10	1.0×10^7	< 111	> 4.96	$> 1.6 \times 10^6$
Human Norovirus	10	1.0×10^7	< 111	> 4.96	$> 1.6 \times 10^6$
Hepatitis A Virus	10	1.0×10^7	< 111	> 4.96	$> 1.6 \times 10^6$

Adsorption of parasites onto charge-modified carbon nanofibers

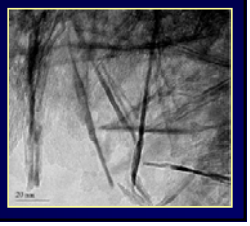
Organism	Influent Volume (L)	Influent titer (per liter)	Effluent titer (per liter)	Log_{10} Reduction	Adsorbed microbes (per gram carbon)
Cryptosporidium parvum oocysts	10	1.0×10^6	< 100	> 4.00	$> 1.6 \times 10^4$

Argonide NanoCeram® Virus Sampler Filters

- Inexpensive: $40/filter
  (1MDS: $175/filter)
- High flow rates (up to 19 L/min)

NanoCeram® Virus Filters

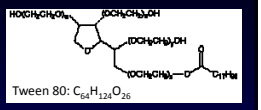
- Alumina nanofibers [$Al(OH)_2$] on microglass fiber matrix
- Electropositive, non-woven, pleated, average pore size = 0.2μm
- Pre-sterilized
- Effective for fresh, brackish, seawater
- pH 5-10; Temps. 4-50°C



Experimental Protocol

- Test organism added to dechlorinated tap water at 2.0×10^8 pfu / 20 L in a pressure vessel.
- Pressure applied (~ 2 p.s.i.) = flow rate of 2.0 L/min.
- Effluent samples collected to determine capture efficiency.
- 450 ml of eluting solution added to the filter housing (30 min hold).
- Eluting solution back flushed through the filter and collected (pH adjusted to 7.5).
- Eluent back flushed a second time.
- Eluent assayed for virus recovery.

Elution Methods

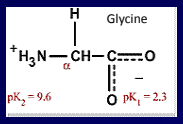


Hydrophobic interactions:

- Surfactants (Tween 80)
- Chaotropic agents (Cl_3CCO_2Na)

Electrostatic interactions:

- High pH (Beef extract, glycine, NaOH)
- Salt solutions
- Sodium polyphosphate (highly negatively charged)



MS2 Phage Recovery From NanoCeram® Filters

Eluting Solution					Elution Efficiency (% Recovery)
3% (w/v) Beef Extract	0.05 M Glycine	0.01 M Phosphate Buffer	0.3% (v/v) Tween 80	1.0% Sodium polyphosphate	
✓					21.9
✓	✓				24.6
	✓	✓			51.8
		✓	✓		39.2
	✓	✓		✓	55.9

Secondary Concentration Step

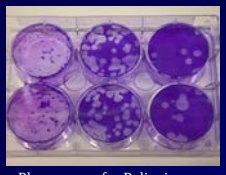
Volume reduction - centrifuge tube ultrafiltration (Vivaspin concentrator)

Reduces volume ~1000-fold (from 150 ml to ~150 µl)

Virus Assay

- Quantify number of viruses recovered using plaque-forming assay or tissue culture infectious dose 50 assay ($TCID_{50}$).

PLC cell monolayer infected with Adenovirus



Plaque assay for Poliovirus on BGM cell monolayers

Recovery of Microorganisms from NanoCeram® Filters

Test Organism	pH	Filter Retention (%)	Elution Efficiency (%)	Method Efficiency After Concentration Step (%)
MS2 bacteriophage	9.3	99.95	55.9	54.6
Poliovirus 1	9.3	99.92	41.4	25.0
Adenovirus 2	9.3	99.90	36.8	22.2
Coxsackie B5	9.3	99.89	51.7	31.9
Echovirus 1	9.3	99.65	107	163.5
Escherichia coli	9.3	99.997	6.7	ND

Method Advantages

- Much lower cost ($40 for NanoCeram® filters vs. $175 for 1MDS filters)
- No organics used in the elution step
- Reduced volume (~ 200 µl vs. ~ 20 ml)
- Higher efficiencies than those reported for some enteric viruses.

Future Work

- Comparison to existing methods in a field study collecting surface water samples in Arizona, Michigan, and Mexico:
  - 1MDS filters, ultrafiltration
  - Adenoviruses, enteroviruses
  - cell culture, polymerase chain reaction
- Evaluate physical methods for recovery of parasites (Microsporidia) from NanoCeram® filters.

Acknowledgments

Luisa Ikner
Marcela Soto

Questions?

Development and Evaluation of an Innovative System for the Concentration and Quantitative Detection of CCL Pathogens in Drinking Water

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David Walt
Udi Zuckerman

Tufts University Cummings School of Veterinary Medicine
Grafton, Massachusetts

EPA 5/20/2009

Tufts Cummings School of Veterinary Medicine
The Division of Infectious Diseases
North – Grafton, MA

Overview

- Milestones of the Continuous Flow Centrifugation methodology (CFC) developed at Tufts
- Objectives of the current STAR award 2006 – 2009
- Progress: new automated method/equipment for multiple waterborne pathogens
- Future tasks
- Acknowledgements

Tier 2 mean results of the CFC compared to Method 1623 criteria

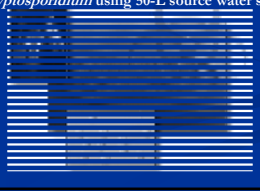
Matrix/Organism	Method 1623 Acceptable Range of Mean Recovery (%)	CFC Study Mean Recovery (%)
Reagent Water *Cryptosporidium*	21-100	42.5
Giardia	17-100	47.2
Source Water *Cryptosporidium*	13-111	37.4
Giardia	15-118	32.6

PCFC Approved by EPA as a Standard Concentration Method

EPA United States Environmental Protection Agency

Method 1622: *Cryptosporidium* in Water by Filtration/IMS/FA December 2005

Changes in the December 2005 Version of the Method
The method was revised again in 2005 to support promulgation of EPA's Long Term 2 Enhanced Surface Water Treatment Rule. Changes incorporated into the June 2003 version include:
Nationwide approval of the use of portable continuous-flow centrifugation as a modified version of the method. The product met all method acceptance criteria for *Cryptosporidium* using 50-L source water samples.



Cont..

CFC 200

2005 - the CFC 200 and 625B bowl became commercially available

2007 – Under the second EPA STAR award

1st automated CFC prototype

Objectives for 2006-2009

- Simultaneous concentration of representative microorganisms from each group of the CCL list
- Validation of the concentration methodology
- Detection and quantitative identification of the CCL list using multiplex miniaturized fiber optic bead microarrays coupled with a compact scanner
- Side by side comparison of this detection methodology with EPA standard methods

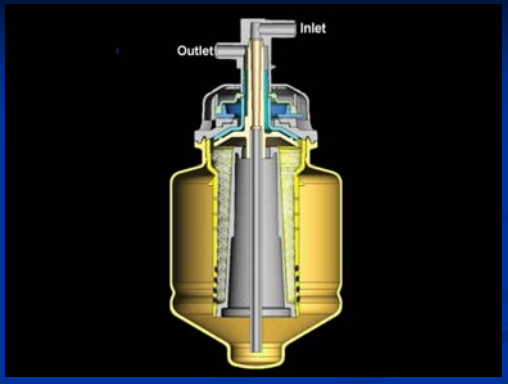
Expanding the CFC methodology beyond protozoa concentration

- Design of a new multiple pathogens bowl
- Design of a portable computerized concentration/elution equipment
- Design of a disposable tubing kit
- Choosing the programming software
- Testing variable operating protocols

How does it work?

- Filtration components are based on size exclusion which is prone to clogging and the overall procedure is labor intensive and expensive
- The new automated CFC methodology employs centrifugal force to sediment the protozoa and bacteria inside the bowl with minimal clogging problems.
- The modified bowl allows the "particle-free sample" to flow through the positive charged component in the core and the viruses are adsorbed by the positive electrostatic forces
- Elution buffers are injected sequentially where the trapped protozoa/bacteria first, then the viruses second, are dislodged and the concentrates are delivered to two separate sterile bags.

New bowl for simultaneous pathogen concentration



Automated CFC protocol

Water Sample 10 – 50 L

↓

Concentration: Assemble the modified bowl/tubing harness Turn on the CFC, select operation mode Protozoa, bacteria and viruses are concentrated inside the bowl

↓

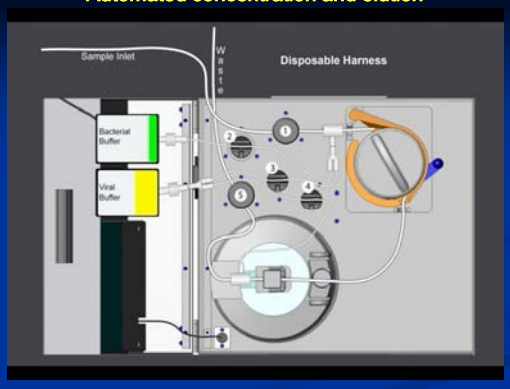
Elution: Protozoa/bacteria buffer is injected, the bowl goes through shaking cycles, the concentrate (~200ml) is delivered to a sterile infusion bag. Virus buffer with a neutral charge is then injected, the bowl goes through rinsing cycles, the concentrate (~20ml) is delivered to a 2nd bag

Detection: The first concentrate is divided into bacteria and protozoa aliquots, and together with the virus concentrate are then processed for detection using standard or rapid methods

Spiking experiments using the automated CFC

Disposable Kit

Automated concentration and elution



Recovery efficiency of the automated CFC with 10 L tap water samples spiked with multiple microorganisms

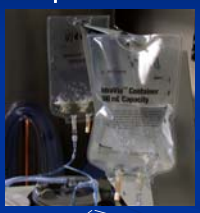
- *C. parvum* were spiked and the oocysts detected from the concentrate using method 1623
- MS2 bacteriophages (ATCC 15597-B1) were spiked and detected from the concentrate using the agar overlay method (the host was *E. coli* 1559)
- *B. anthracis* spores (kanamycin resistant strain, sterne) detected by MF

Recovered concentrates and detection methods

Virus concentrate

Agar overlay procedure for MS2 phage

Bacteria/protozoa concentrate



EPA Method 1623 for *C. Parvum*

Vacuum filtration and media growth for *B. anthracis*

Recovery of *C. parvum* oocysts, *B. anthracis*, and MS2 bacteriophages from 10L tap water samples using an automated CFC and a modified bowl (9,000rpm & 0.5 liter/min)

Spiked samples volume (L) (# replicates)	*C. parvum* spike dose (mean +/- SD)	*C. parvum* percent recovery (mean +/- SD)	*B. anthracis* spike dose (CFU mean +/- SD)	*B. anthracis* percent recovery (mean +/- SD)	MS2 bacteriophages spike dose (mean +/- SD)	MS2 bacteriophages recovery (mean +/- SD)
10 (7)	100 +/- 2.5	40 +/- 12.2	23.3 +/- 4.6	43.6 +/- 16.4	$2.6*10^7$ +/- $1.3*10^7$	48.1+/-28.2

Detection and integration

- We have concentrated on the detection of DNA isolated from *E. coli* as a model system. We have demonstrated the detection of PCR amplicons from three virulence genes using multiplexed bead-based microarrays.
- We expanding the protocol and microarray to include all bacteria and viruses listed as CCL3 candidates as listed

CCL3 candidates

Caliciviruses
Campylobacter jejuni
Entamoeba histolytica
Escherichia coli (0157)
Helicobacter pylori
Hepatitis A virus
Legionella pneumophila
Naegleria fowleri
Salmonella enterica
Shigella sonnei
Vibrio cholerae

Accomplishments

- A prototype automated pathogen concentrator was designed and constructed
- This includes modification of the hardware and of the disposables
- The device weighs: 45lb; 110/220AC/12 VDC
- Capable of simultaneous concentration of protozoa (*Cryptosporidium*), bacterial spores (*B. anthracis*) and MS2 from volumes of 10-50L
- Computer programmable PLC capable of handling numerous automated protocols

Pathogen	Spike dose	Recovery (%) for 10L N = 12	Recovery (%) for 50L N = 2
protozoa	100±1	40±0.06	~ 40
spores	50±5	34±0.14	~ 30
MS2	10^5	43±0.3	~ 50

The next phase

- Walt's lab is currently working on the bioinformatix of the CCL list for the microarray detection: this will be completed over the next 12 months
- Once the detection platform is complete, the automated CFC spiked concentrates will be applied and qunatitated
- The detection will be compared with currently approved standard methods
- Ideally this approach should be evaluated by water testing labs – filed testing, as was done for *C. parvum* and Giardia
- Evaluate the technology as a continuous monitoring system

Acknowledgements

- EPASTAR program (RD 83300301) which is funding this work
- Haemonetics for technical and material support over the past 10 years
- Staff of the Division of Infectious Diseases for technical support

MICHIGAN STATE UNIVERSITY

On-chip PCR, Nanoparticles, and Virulence/Marker Genes for Simultaneous Detection of 20 Waterborne Pathogens

U.S. EPA Workshop on Innovative Approaches for Detecting Microorganisms and Cyanotoxins in Water

Philadelphia, PA

May 20, 2009 3:15 PM

Syed A. Hashsham

Edwin Willits Associate Professor

Volodymyr Tarabara

Assistant Professor

James M. Tiedje

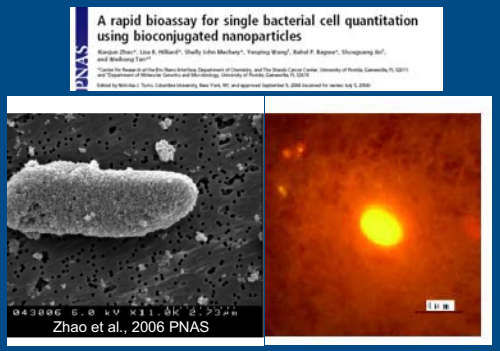
University Distinguished Professor and Director, Center for Microbial Ecology

Department of Civil and Environmental Engineering and Center for Microbial Ecology
Michigan State University

Objectives

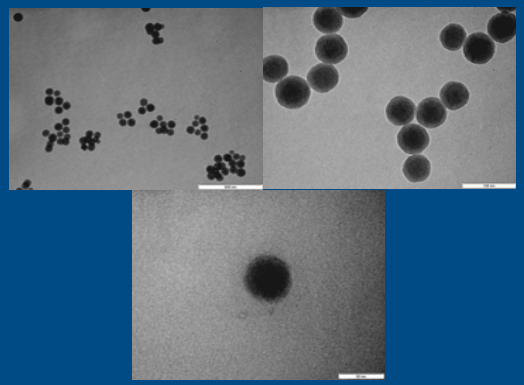
1. **Reducing the Time to Detect Growth using Dye-doped Nanoparticles**
2. **On-chip PCR based Detection of 20 Pathogens**
3. **Enhancement in Sample Concentration by Cross-flow Filtration**

1. Dye-doped nanoparticle-based detection of growth



SEM and Fluorescence image of *E. coli* 0157:H7 incubated with antibody conjugated dye doped nano-particles

Nano-particles for monitoring of growth



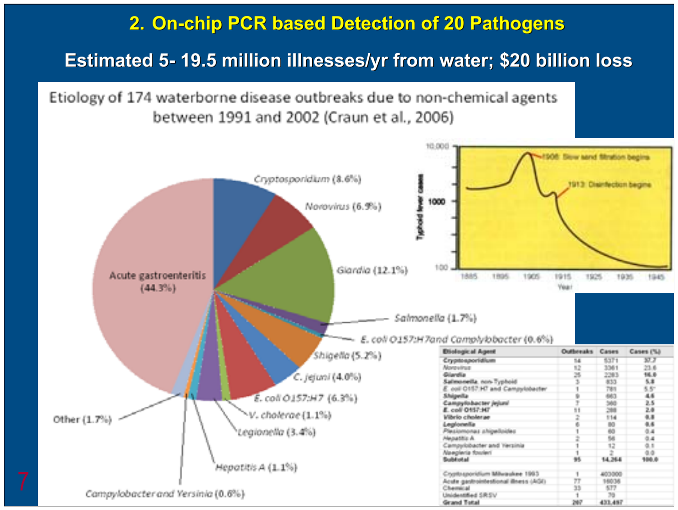
TEM images of dye-doped silica NPs. Particle size is about 55 nm
(Yang et al., Submitted, 2007)

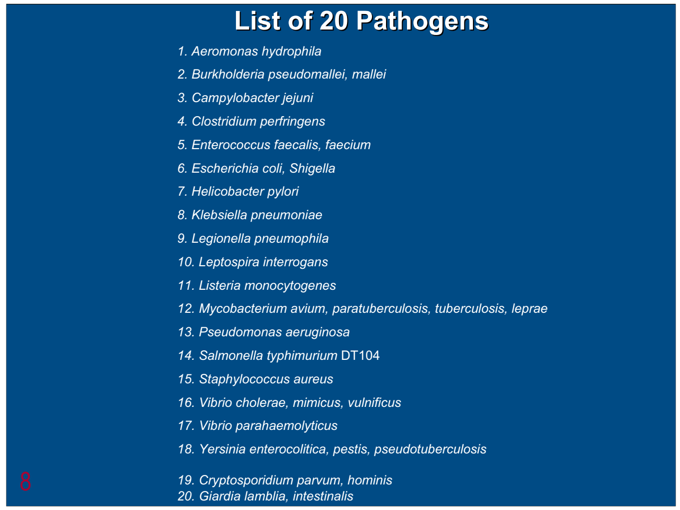
Growth curve by plate count, real time PCR, absorbance and dye doped NP assay

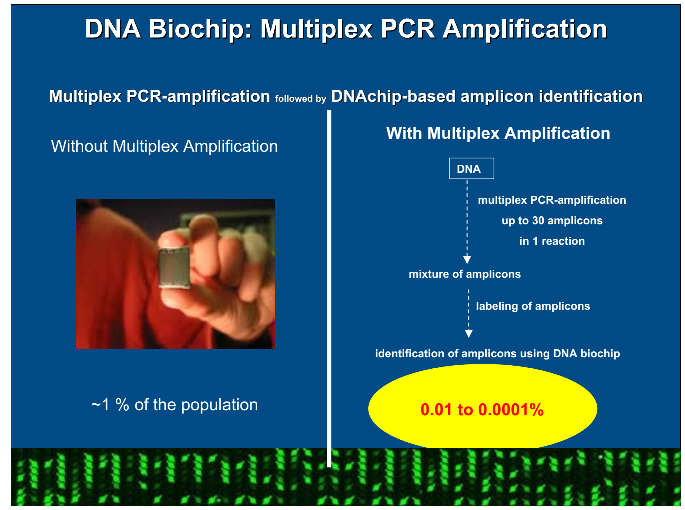
Contact the presenters

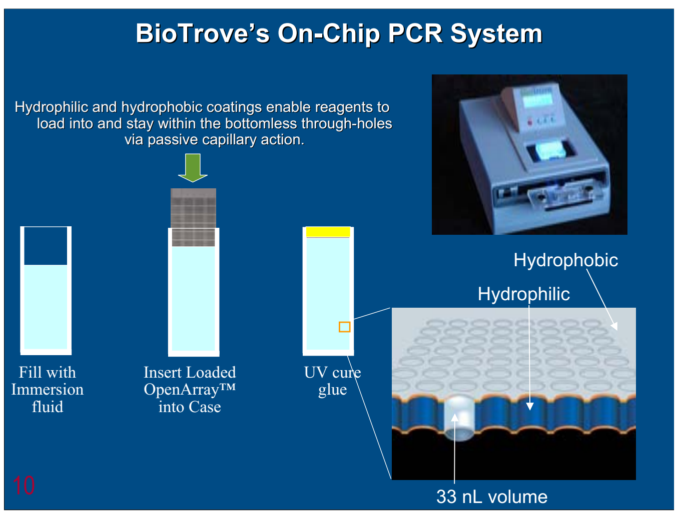
Time taken to determine the increase in growth by various methods

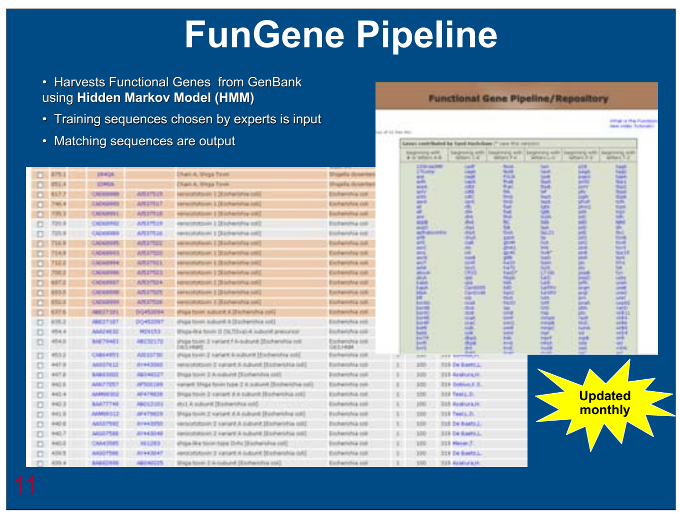
Contact the presenters

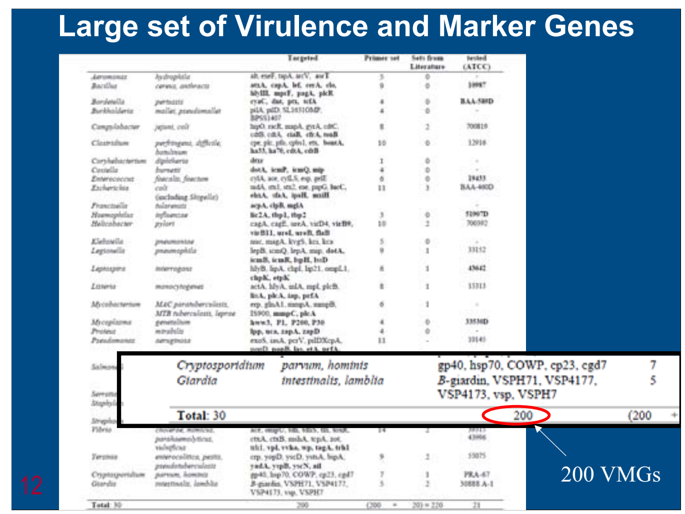

2. On-chip PCR based Detection of 20 Pathogens
Estimated 5- 19.5 million illnesses/yr from water; $20 billion loss
Etiology of 174 waterborne disease outbreaks due to non-chemical agents between 1991 and 2002 (Craun et al., 2006)
Cryptosporidium (8.6%)
Norovirus (6.9%)
Giardia (12.1%)
Acute gastroenteritis (44.3%)
Salmonella (1.7%)
E. coli O157:H7 and Campylobacter (0.6%)
Shigella (5.2%)
C. jejuni (4.0%)
E. coli O157:H7 (6.3%)
V. cholerae (1.1%)
Legionella (3.4%)
Hepatitis A (1.1%)
Other (1.7%)
Campylobacter and Yersinia (0.6%)
1908: Slow sand filtration begins
1913: Disinfection begins
Typhoid fever cases
Year
7


List of 20 Pathogens
1. Aeromonas hydrophila
2. Burkholderia pseudomallei, mallei
3. Campylobacter jejuni
4. Clostridium perfringens
5. Enterococcus faecalis, faecium
6. Escherichia coli, Shigella
7. Helicobacter pylori
8. Klebsiella pneumoniae
9. Legionella pneumophila
10. Leptospira interrogans
11. Listeria monocytogenes
12. Mycobacterium avium, paratuberculosis, tuberculosis, leprae
13. Pseudomonas aeruginosa
14. Salmonella typhimurium DT104
15. Staphylococcus aureus
16. Vibrio cholerae, mimicus, vulnificus
17. Vibrio parahaemolyticus
18. Yersinia enterocolitica, pestis, pseudotuberculosis
19. Cryptosporidium parvum, hominis
20. Giardia lamblia, intestinalis
8

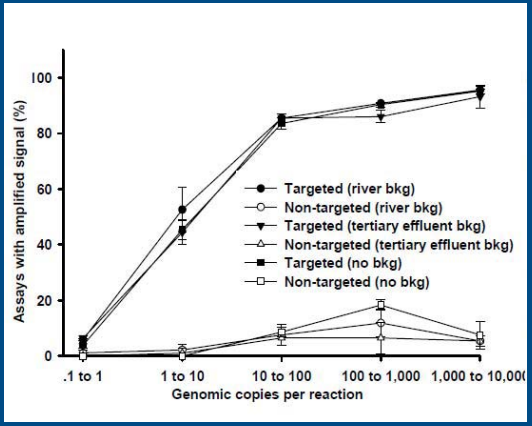

DNA Biochip: Multiplex PCR Amplification
Multiplex PCR-amplification followed by DNAchip-based amplicon identification
Without Multiplex Amplification
With Multiplex Amplification
DNA
multiplex PCR-amplification
up to 30 amplicons
in 1 reaction
mixture of amplicons
labeling of amplicons
identification of amplicons using DNA biochip
~1 % of the population
0.01 to 0.0001%


BioTrove's On-Chip PCR System
Hydrophilic and hydrophobic coatings enable reagents to load into and stay within the bottomless through-holes via passive capillary action.
Fill with Immersion fluid
Insert Loaded OpenArray™ into Case
UV cure glue
Hydrophobic
Hydrophilic
33 nL volume
10


FunGene Pipeline
• Harvests Functional Genes from GenBank using Hidden Markov Model (HMM)
• Training sequences chosen by experts is input
• Matching sequences are output
Functional Gene Pipeline/Repository
Updated monthly
11


Large set of Virulence and Marker Genes
Cryptosporidium parvum, hominis gp40, hsp70, COWP, cp23, cgd7 7
Giardia intestinalis, lamblia B-giardin, VSPH71, VSP4177, VSP4173, vsp, VSPH7 5
Total: 30 200 (200
200 VMGs
12

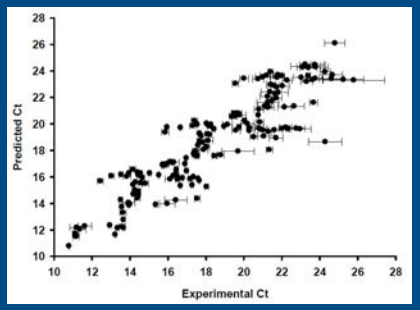
LOD: 10 copies!



(Stedtfeld et al., 2008)

13

Quantification without standard curves?



gs = genome size of target organism

GC = GC content of target organim's genome

Tm_7 = theoretical melt temperature of last 7 bases on primer 3' end

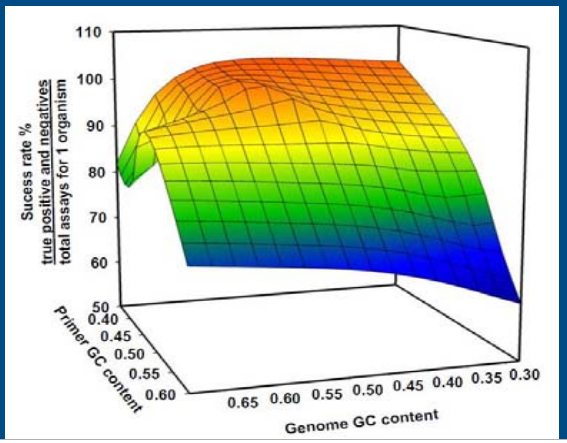
al = amplicon length

Predicted Ct versus theoretical Ct based on empirical equation

(Stedtfeld et al., 2008)

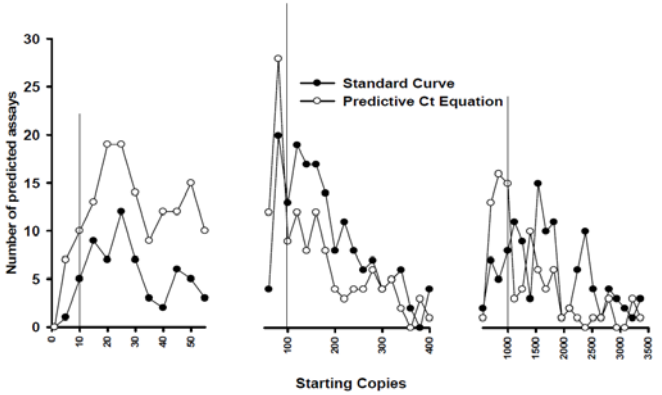
14

High GC Content and Larger Genomes: More Problematic



15

Larger difference at lower copy numbers



Hand-held Gene Analyzer

Contact the presenters

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Effect of Exposure Time, Path Length, and DNA Concentration on Signal Intensity

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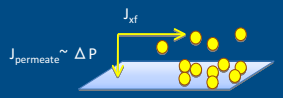
3. Enhancement in Sample Concentration by Cross-flow Filtration

- **Goals:** Increasing
  1) Rate of concentration ($J_{permeate}$)
  2) Recovery
  3) Reproducibility



- **Approach:**

➢ Hydraulic management (Goals 1 & 2)
- ΔP ↗ → $J_{permeate}$ ↗
- J_{xf} ↗ → $J_{permeate}$ ↗ and recovery ↗

➢ Preparation of reproducible non-adhesive membrane (Goals 2 and 3):
- Non adhesive surface → recovery ↗
- Controlled approach to membrane blocking → reproducibility ↗

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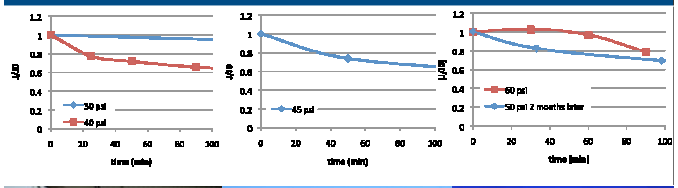
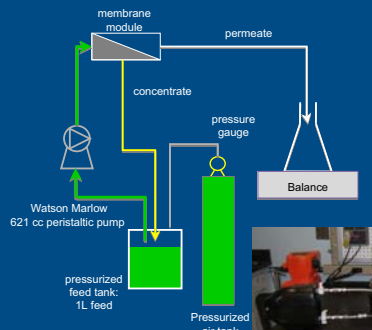
Pump Evaluation: Reduction in Cross-flow over Time



Influence of ΔP and $J_{crossflow}$ on microorganism recovery: Experimental setup

Membrane: PES 30kDa, blocked with 5% calf serum, 44 cm²



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Rate of Sample Concentration

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* **Amount of water filtered in 30 min normalized to 1 m² of membrane surface area**

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Influence of ΔP and $J_{crossflow}$ on Bacteriophage Recovery

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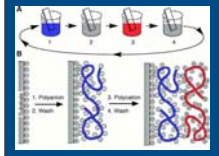
Design of non-adhesive surface

- Protein Blocking of the membrane:

  May not always be "appropriate or practical due to concern related to the amount of time needed (…) and potential for microbial contamination"

  Hill et al. 2005

➢ New approach to membrane blocking



Source: G. Decher, 1997

- reproducible, non-adhesive coatings based on multilayer polyelectrolyte films
- fast and and straightforward coating procedure
- design flexibility (charge, hydrophilicity)
- have been shown to reduce adhesion of bacteria, mammalian cell and proteins
- recoverable coating

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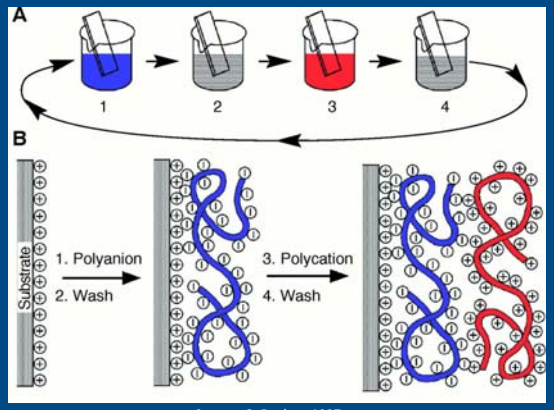
PEM deposition procedure

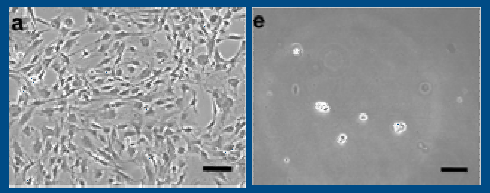


Source: G. Decher, 1997

25

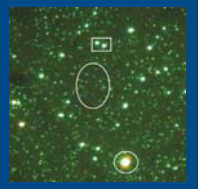
Potential of Polyelectrolytes as Blocking Agents

- Fibroblast adhesion before and after deposition of PEM (PAA/PAAm)



Yang et al. 2003

- Some polyelectrolytes inhibit phage infectivity of bacteria (plaque assay cannot be used)
- Epifluorescence and PCR are being evaluated as alternative methods of quantifying viruses



Patel et al. 2007

26

Summary

1. **NP-based assay faster but expect to be busy**
2. **On-chip PCR: efficient screening tool, for samples that will result in 10 copies**
3. **Sample concentration speed can be considerably improved with higher pressure (8 fold to 150 L/30 min-m^2)**
4. **Improvements in blocking the filters: ongoing**

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Acknowledgements

This research is funded by U.S. EPA - Science To Achieve Results (STAR) Program
Grant # R833010

Michigan Economic Development Corporation's 21st Century Jobs Fund

Doctoral candidates:

Robert Stedtfeld	Most chip related experiments
Elodie Pasco	Membrane Filtration studies
Tiffany Stedtfeld	Validation
Dieter Tourlousse	Sample Processing/DNA Biochip
Farhan Ahmad	micro-PCR Image Analysis
Yu Yang	Nanoparticles

PIs: **Syed Hashsham, Volodymyr Tarabara, and James Tiedje**

28

Rapid and Quantitative Detection of *Helicobacter pylori* and *E. coli* O157 in Well Water Using a Nano-Wired Biosensor and QPCR

2009 U.S. Environmental Protection Agency Workshop on Innovative Approaches for Detecting Microorganisms and Cyanotoxins in Water
May 20-21, 2009, Philadelphia, PA

E.C. Alocilja, J.B. Rose, E. Dreelin
Shannon McGraw, Michelle Packard, Jongseol Yuk,
Lauren Bul, and Teresa Brinks
Michigan State University

Outline

- Hypothesis
- Results by objectives
- Summary of results
- Future work

Hypothesis

- A disposable biosensor and qPCR can be combined seamlessly to develop a unique biosensor-qPCR as a tool for near real-time determination of contaminant occurrence in drinking water.

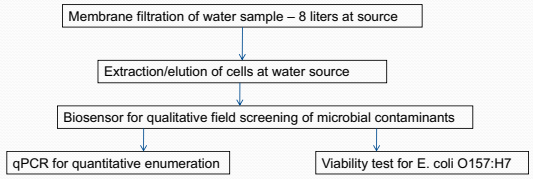
Objectives

- Develop a protocol for processing water samples for the biosensor and qPCR.
- Assess the performance of the biosensor and qPCR for sensitivity, specificity, recovery, and false positives/negatives of detection and enumeration for *E. coli* O157:H7 and *H. pylori* in groundwater samples from the field.
- Develop a method for detecting and enumerating *E. coli* O157:H7 and *H. pylori* by qPCR using bacteria isolated and screened by the biosensor system.
- Validate a method for testing viability of *E. coli* O157:H7.

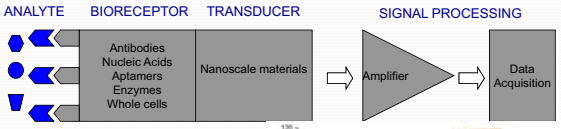
Highlight of Results

- Developed a novel target extraction system using an electrically active magnetic nanoparticles.
- Developed a protocol for use of automated DNA extraction and evaluated it in difficult samples.
- Developed a data base on CFU vs qPCR units for *E.coli* and Enterococci, and will be adding in the data from each sample for the 0157.
- E. coli O157:H7 biosensor has been tested in pure and seeded water samples.
- Viability test has been developed; sensitivity and specificity were evaluated.

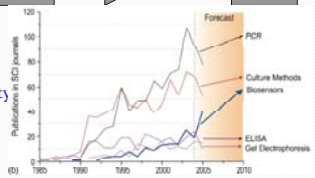
Flowchart of Research Plan



Biosensor



- Advantages:
  - Rapid detection time
  - High sensitivity and specificity
  - Compatible with data processing technologies
  - Can be ruggedized



Lazcka et al., Biosens. Bioelectron., 2007

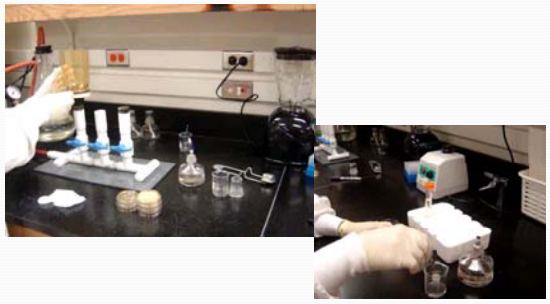
Real-Time Quantitative PCR (qPCR)

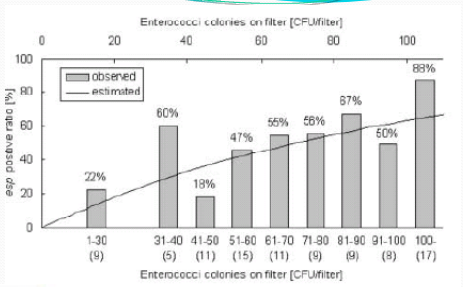
- Detects PCR product fluorescently in each well plate.
- Fast PCR screening without gels.
- Quantifies amount of PCR product at each cycle.
- Detects presence or quantify fraction of sample made up by particular species using species specific primers.
- Uses threshold detection for relative abundance.

Results By Objectives

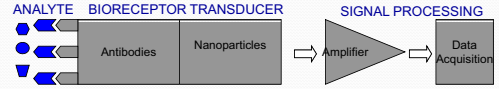
- **Develop a protocol for processing water samples for the biosensor and qPCR.**
- Assess the performance of the biosensor and qPCR for sensitivity, specificity, recovery, and false positives/negatives of detection and enumeration for *E. coli* O157:H7 and *H. pylori* in groundwater samples from the field.
- Develop a method for detecting and enumerating *E. coli* O157:H7 and *H. pylori* by qPCR using bacteria isolated and screened by the biosensor system.
- Validate a method for testing viability of *E. coli* O157:H7.

Membrane Filtration (MF) & Enrichment

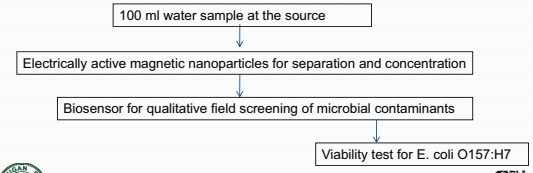




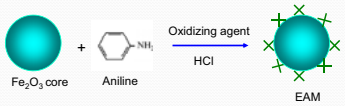
Using EAM Nanoparticles for Target Extraction



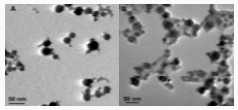
- Electrically active magnetic nanoparticles (EAM) functionalized with antibodies



Iron oxide-polyaniline core/shell → EAM



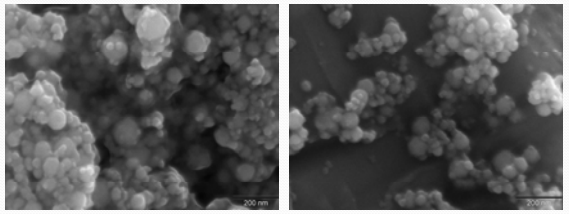
- Unique electronic structure and flexible electrical properties of protonated polyaniline
- Magnetic properties from the core
- Simple and low cost preparation
- Excellent environmental stability



TEM images of (left) unmodified Fe_2O_3 NPs and (right) electrically active magnetic NPs.

Characterization of EAM

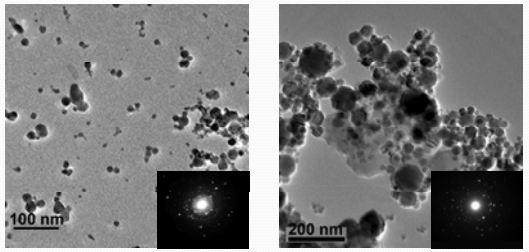
Scanning Electron Microscopy Images



EAM Nanoparticles (1:0.4) Iron oxide Nanoparticles

Characterization of EAM

Transmission Electron Microscopy and Electron Diffraction Images

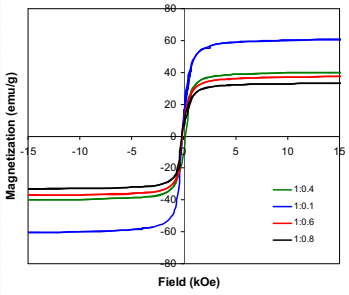


1:0.1 EAM NPs 1:0.4 EAM NPs

XRD shows EAM is crystalline.

Magnetic Measurement of EAM

Experimental hysteresis measurements at 300K



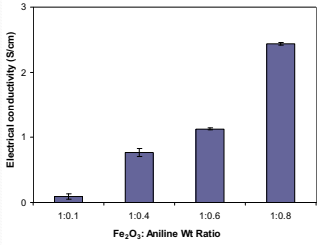
γ-Fe_2O_3 nanoparticles: aniline monomer weight ratio was varied as 1:0.1, 1:0.4, 1:0.6, and 1:0.8.

Magnetic characterization

γ-Fe_2O_3: Aniline Wt. Ratio	Coercivity (300K) Oe	Retentivity (300K) emu/g	Saturation Magnetization (emu/g)
1:0.1	180	15.3	61.1
1:0.4	180	9.57	40.3
1:0.6	180	9.48	37.7
1:0.8	180	9.18	33.5

Low coercivity and retentivity values → EAMs are in the ferromagnetic regime.

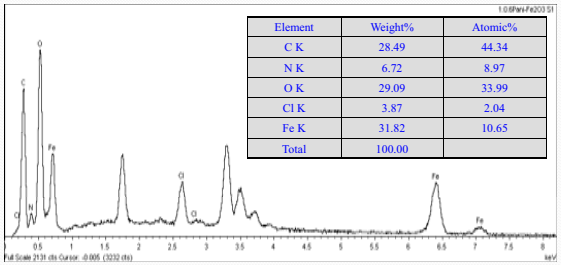
Electrical conductivity of EAM



Four point probe measurements in compressed pellets of 2000 microns in thickness.

γ-Fe_2O_3: Aniline Wt. Ratio	Conductivity (S cm^{-1})
1:0.1	0.092
1:0.4	0.768
1:0.6	1.129
1:0.8	2.436
1:0.0	0.000017

Energy dispersive spectroscopy



Element	Weight%	Atomic%
C K	28.49	44.34
N K	6.72	8.97
O K	29.09	33.99
Cl K	3.87	2.04
Fe K	31.82	10.65
Total	100.00	

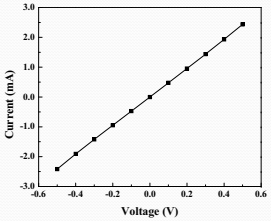
1:0.6 EAM Nanoparticle

Electrical Characterization of EAM

EAM follows ohmic behavior.

Ohm's law:

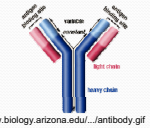
I = V/R



1:06 ratio

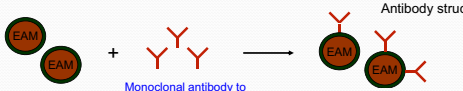
Ab-EAM for Cell Capture

- Method: Physical adsorption *(Muhammad Tahir et.al, 2003)*
  - Combine EAM nanoparticles with monoclonal antibodies to target cell in PBS solution
- Conditions:
  - Room temperature incubation
  - Time: 45 min



www.biology.arizona.edu/.../antibody.gif

Antibody structure

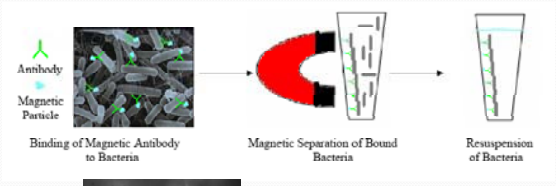


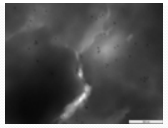
Monoclonal antibody to Target cell

Antibody Modified EAMs

Immuno-EAM Bacterial Separation

- Monoclonal antibody specific to *Escherichia coli O157:H7* conjugated to EAM nanoparticles
- Bound bacteria separated using a magnetic separator and resuspended in deionized water





TEM image of polyaniline conjugated with antibodies (labeled with gold nanoparticles to confirm binding of Ab on polymer surface).

Experiments for immuno-EAM capture -- for 10^6 cfu/ml

- Incubation time: 15, 30, 60 min
  - → 30 min had most cell capture
- Antibody concentration: 0.1, 0.25, 0.5, 1.0 mg/ml
  - → 0.5 mg/ml had most cell capture
- EAM concentration: 10, 20 25 mg.ml
  - → 10 mg/ml had the most cell capture

Capture efficiency for E. coli O157:H7

- Cell capture was confirmed by plating:

Solution	Count of Captured Cells	Cell Count in Original Culture
10^{-5} dilution of pure culture (10^4 CFU/ml)	10,880 CFU/ml (10^4 CFU/ml)	**1.088×10^9 CFU/ml**
10^{-6} dilution, cell conjugate (10^2 CFU/ml)	10 CFU/ml (10^1 CFU/ml)	**4.0×10^8 CFU/ml**

- Observation: Capture process decreased cell count by less than a factor of 10.

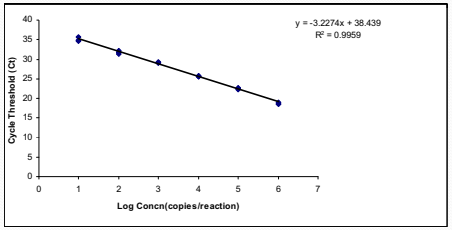
Results By Objectives

- Develop a protocol for processing water samples for the biosensor and QPCR.
- **Assess the performance of the biosensor and qPCR for sensitivity, specificity, recovery, and false positives/negatives of detection and enumeration for *E. coli* O157:H7 and *H. pylori* in groundwater samples from the field.**
  - **qPCR**
- Develop a method for detecting and enumerating *E. coli* O157:H7 and *H. pylori* by QPCR using bacteria isolated and screened by the biosensor system.
- Validate a method for testing viability of *E. coli* O157:H7.

Primers and probes for the qPCR assays

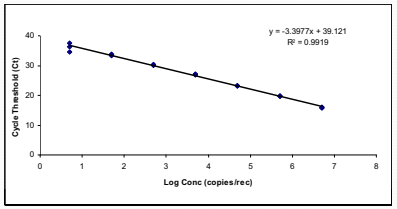
Organisms	Target gene	Primer/Probe	Reference
E.coli O157	uidA	5'CAATGGTGATGTCAGCGTT3' 5'ACACTCTGTCCGGCTTTTG3' HEX- CAACTGGACAAGGGGCACCA GC--BBQ	Developed by this study
E.coli	uidA	5'CAATGGTGATGTCAGCGTT3' 5'ACACTCTGTCCGGCTTTTG3' 6FAM- TTGCAACTGGACAAGGCACCA GC--BBQ	Developed by this study
Enterococci	23SrDNA	AGA AAT TCC AAA CGA ACT TG CAG TGC TCT ACC TCC ATC ATT FAMb-TGG TTC TCT CCG AAA TAGCTT TAG GGC TA-TAMRAc	Frahm *et al*, 2002

Standard curve for *E. coli* assay



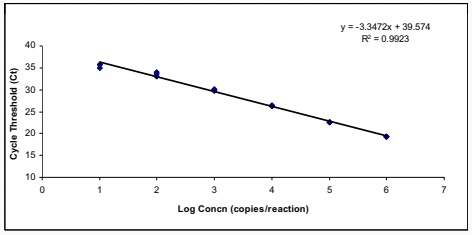
Standard curve for 10-fold serial dilutions of generic *E.coli* uidA gene. Linear regression analysis shows an R^2 of 0.995, a slope of -3.22 and an intercept of 38.439

Standard curve for *E. coli* O157 assay

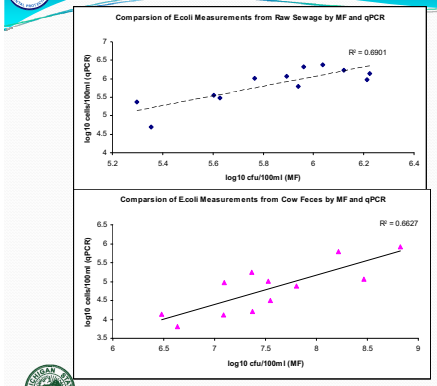


Standard curve for 10-fold serial dilutions of *E.coli* O157 uidA gene. Linear regression analysis shows an R^2 of 0.99, a slope of -3.39 and an intercept of 39.121

Standard curve for Enterococci assay



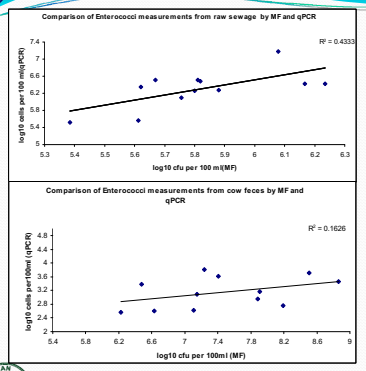
Standard curve for 10-fold serial dilutions of generic enterococci 23SrDNA gene. Linear regression analysis shows an R^2 of 0.992, a slope of -3.34 and an intercept of 39.574



qPCR for E. coli

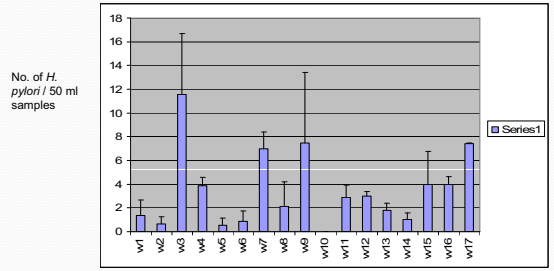
***E.coli*, target**

1 copy of uidA gene per cell
Betaglucuronidase Gene



qPCR for Enterococci

Analysis of VacA gene of *H. pylori* Vs Samples



Raw water from waste water treatment plant at different period of time

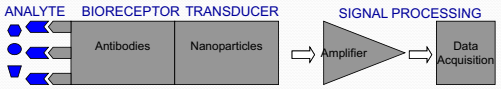
Key Results

- Rapid qPCR methods have been developed for two fecal indicators *E.coli* and Enterococci and two pathogens *Helicobacter* and *E.coli* 0157H7.
- qPCR has been used to detect *Helicobacter* in sewage and detects what is likely the viable non-cultivable state (previous report and publication).
- qPCR is highly correlated to *E.coli* and Enterococci in Sewage but this same assay does not detect all of the species present in manure, either due to interferences or more likely due to specificity of the primers.

Results By Objectives

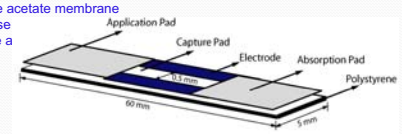
- Develop a protocol for processing water samples for the biosensor and QPCR.
- **Assess the performance of the biosensor and qPCR for sensitivity, specificity, recovery, and false positives/negatives of detection and enumeration for *E. coli* O157:H7 and *H. pylori* in groundwater samples from the field.**
  - **Biosensor**
- Develop a method for detecting and enumerating *E. coli* O157:H7 and *H. pylori* by QPCR using bacteria isolated and screened by the biosensor system.
- Validate a method for testing viability of *E. coli* O157:H7.

Performance of biosensor



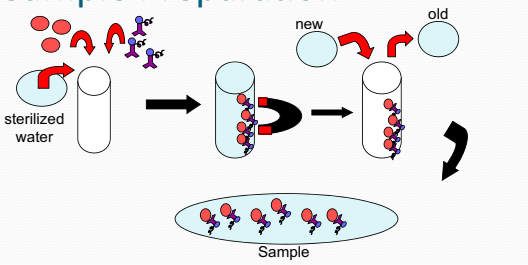
Lateral flow direct-charge transfer biosensor

Application Pad: Cellulose acetate membrane
Capture Pad: Nitrocellulose
Absorption Pad: Cellulose a
Electrode: Silver
Type: Disposable

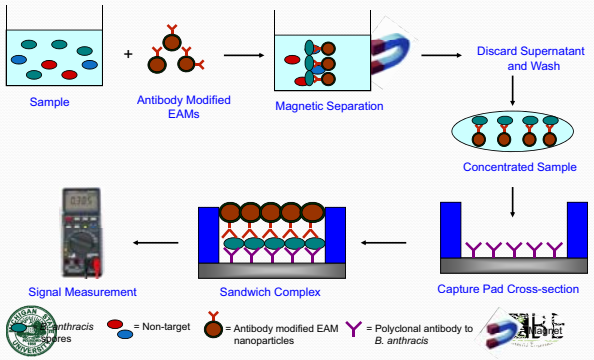


Overall Dimension: 60mm × 5 mm
Electrode Gap: 0.5 mm

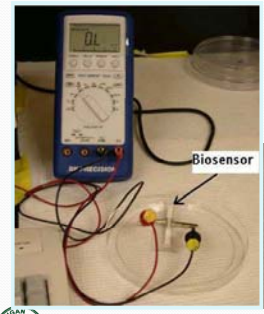
Sample Preparation



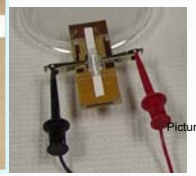
Nano-BEAM DCT biosensor detection schematic



Picture of DCT biosensor



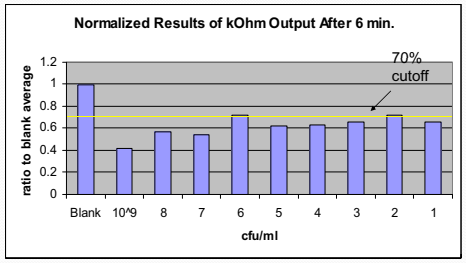
Dimension: 60mm x 5mm



Antibodies and Bacterial Isolates

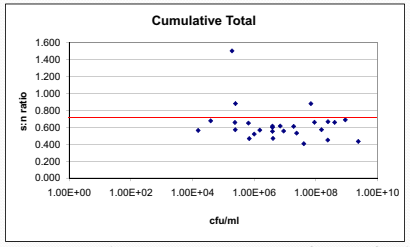
- Antibodies
  - Purified mouse monoclonal anti-*E.coli* O157:H7 (OEM Concepts)
  - Purified goat polyclonal anti-*E.coli* O157:H7 (Kirkegaard & Perry Laboratories Inc.)
- Bacterial Isolate
  - *E.coli* O157:H7 C3000

Results in Pure Culture



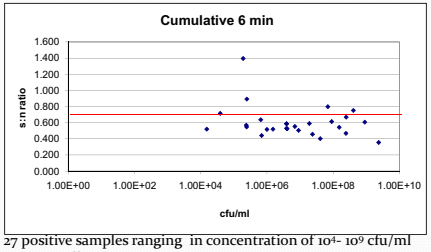
- Data can be analyzed as a positive or negative detection based on concentration averages or individual readings
- Negatives seen at 10^6 and 10^2 cfu/ml; has fewer recorded data points

Seeded Water Samples With Various Cell Concentrations



- 27 positive samples ranging in concentration of 10^4- 10^9 cfu/ml
- 70% cut-off
- 89% true positive; 11% false negative
- Cumulative signal taken 2, 4, & 6 min after sample application

Seeded Water Samples With Various Cell Concentrations

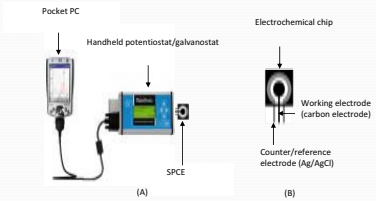


- 27 positive samples ranging in concentration of 10^4- 10^9 cfu/ml
- 70% cut-off
- 81% true positive; 19% false negative
- Detection signal taken 6 min after sample application

Key Results

- Sensitivity studies need to be continued.
- Can not currently quantify the concentration of bacteria in the sample because of observed hook effect due to cell crowding and variances between testing.
- The overall time interval from obtaining a sample to readout with the biosensor is < 20 minutes.
- Biosensor design and parameters need to be modified/improved to minimize false negative.

Proposed Alternative Design: Screen-printed carbon electrode (SPCE) biosensor



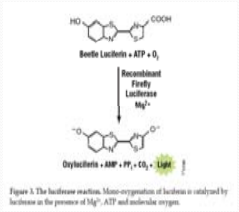
Schematic of the proposed handheld SPCE biosensor (A); Schematic of the 2cm² electrochemical chip containing the screen-printed carbon electrode (black, center) and Ag/AgCl reference and counter electrodes (B).

Results By Objectives

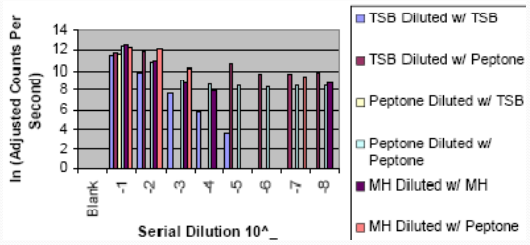
- Develop a protocol for processing water samples for the biosensor and QPCR.
- Assess the performance of the biosensor and qPCR for sensitivity, specificity, recovery, and false positives/negatives of detection and enumeration for *E. coli* O157:H7 and *H. pylori* in groundwater samples from the field.
- Develop a method for detecting and enumerating *E. coli* O157:H7 and *H. pylori* by QPCR using bacteria isolated and screened by the biosensor system.
- **Validate a method for testing viability of *E. coli* O157:H7.**

BacTiter-Glo™ Microbial Cell Viability Assay

- Concentration of *E. coli* C3000 (ATCC #15597) by centrifugation
- Antibody separation
  - Goat-derived, polyclonal, biotinylated antibody (Meridian Life Sciences, Cat# B65109B)
  - Magna-Sphere streptavidin-coated magnetic beads (Promega Cat # Z5481),
- The BacTiterTM Microbial Cell Viability Assay (Promega Cat#C8230)
- Greater numbers of positive results compared to the standard methods
  - Likely due low specificity

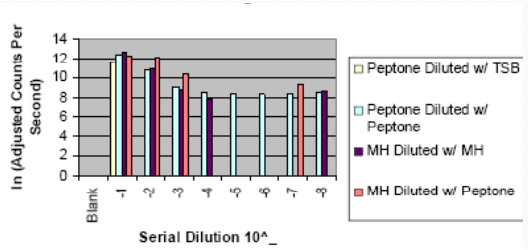


Comparison of Noise Levels as a Result of Diluents

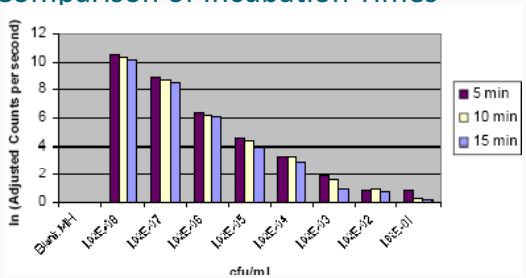


TSB resulted in a loss of detection at a dilution of 10^{-8}, while *E. coli* in both MH broth and peptone water were significantly positive when compared to blanks.

Comparison of Peptone and MH Broth as Diluents

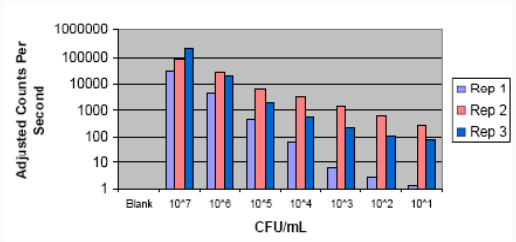


Comparison of Incubation Times



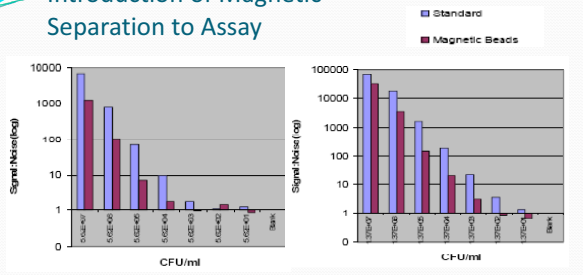
Sensitivity and specificity were insignificantly affected by time

E. coli C3000 Grown in Mueller Hinton Broth



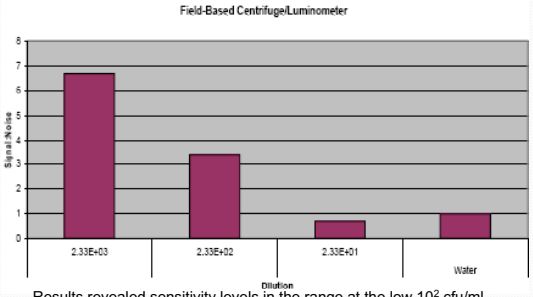
The **BacTiter-GloTM** assay reliably detected live *E. coli* cells at concentrations as low as 10^1 cfu/ml

Introduction of Magnetic Separation to Assay



Although a slight decrease in sensitivity; a detection as low as 1.37 x 10^3 cfu/ml remained possible with magnetic separation

Use of Portable Centrifuge and Luminometer

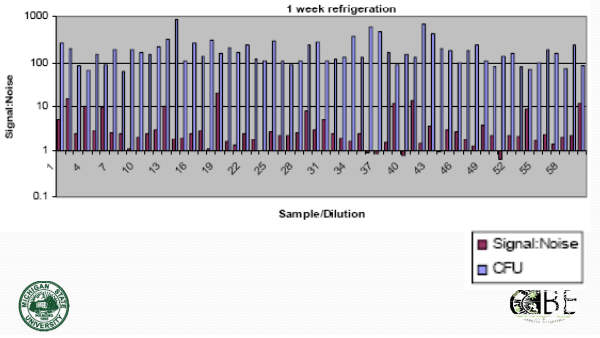


Results revealed sensitivity levels in the range at the low 10^2 cfu/ml

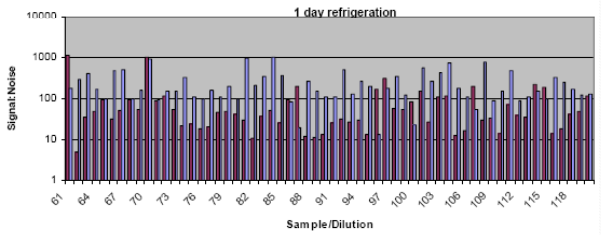
Testing of Environmental Samples

- Sixty river surface water samples obtained from Ingham County Health Department
  - Thirty sites
  - Two sampling dates July 28, 2008 (week 1) and August 4, 2008 (week 2)
  - Both sample cohorts were cultured immediately after collection.
- Gold standard:
  - Samples less than 300 cfu/mL considered negative
  - Equal to or greater than 300 were positive
- Receiver operator curve (ROC) analysis performed using the gold standard as determined by Ingham County Health Department Data.

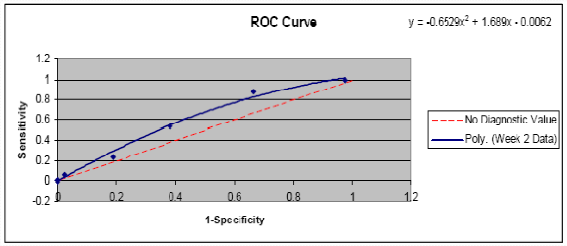
Surface Water Samples Week 1: One Week Refrigeration



Surface Water Samples Week 2: One Day Refrigeration

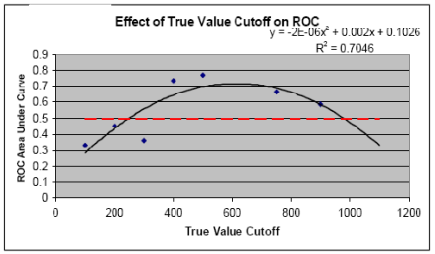


Receiver Operating Characteristic (ROC) Curve Using Cutoff of 300 cfu/mL



The resulting ROC curve (x axis=FPR; y axis=TPR) shows viability assay has little value since the area under the curve is only 0.36. (FPR=false positive rate; TPR=true positive rate)

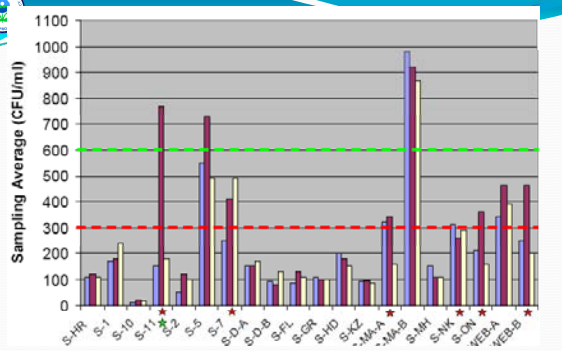
Effect of Gold Standard Cutoff on ROC



The greatest diagnostic value of the assay is noted when the gold standard cutoff is set to approximately 600 cfu/mL; the area under the curve has approximately 0.76

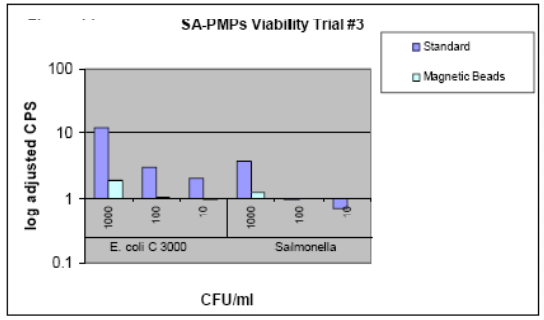
Adoption of Gold Standard Set to 600 cfu/mL

- Cutoff level of 300 cfu/mL
  - 6 sampling sites with contradicting results
  - Contradiction between sampling location (left, center or right)
- Cutoff level of 600 cfu/mL
  - Decreased number of contradicting sites to one
  - Previously positive results now negative

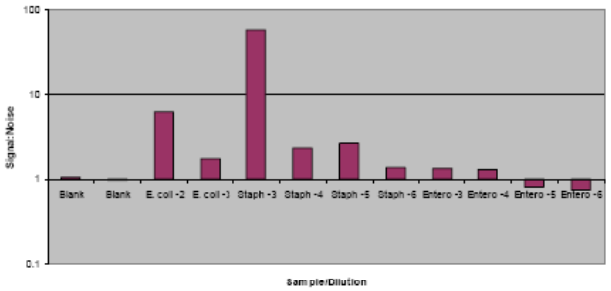


Comparison of current and proposed cutoff levels on week 2 results: Red star indicates sites with contradicting results using the 300 cfu/mL cutoff; green star indicates contradiction with the 600 cfu/mL cutoff.

Specificity Testing: *E. coli* vs. *Salmonella*



Specificity Testing: *E. coli vs. Salmonella, S. Aureus, and Enterococcus*



Alternate Approach to Viability Test

A: Chemical-grade ATP:

$$\text{Glucose} + \text{ATP} \xrightarrow{\text{Hexokinase}} \text{Glucose 6-Phosphate} + \text{ADP} + H^+$$

(Detectable change amperage increase)

B: Hexokinase-bound *E. coli O157:H7*:

$$\text{Glucose} + \text{ATP} \xrightarrow{\text{Hexokinase}} \text{Glucose 6-Phosphate} + \text{ADP} + H^+$$

(Detectable change amperage increase)

C: Negative Controls

$$\text{Glucose} + \text{ATP} \longrightarrow \text{Glucose} + \text{ATP}$$

(No amperage increase)

Output: Papers and Thesis

- **Peer-reviewed Publications:**
  - Yuk, J.S., Jin, J.H., Alocilja, E.C., and Rose, J.B. 2009. Performance enhancement of polyaniline-based polymeric wire biosensor. Biosensors and Bioelectronics Journal 24(5): 1348-1352 (available online at http://dx.doi.org/10.1016/j.bios.2008.07.079 in 2008).
  - Yuk, J.S. and Alocilja, E.C. 2009. Electrical characterization of magnetic polyaniline and bio-conjugated magnetic as molecular biowires. Sensors & Actuators: B. Chemical (in review).
- **Thesis:**
  - Arun Nayak, MS 2008; Stability And Quantitative Surveillance Of *Helicobacter pylori* And *Campylobacter jejuni* In Environmental Waters By Real Time qPCR.

Output: Presentations

- **Presentations:**
  - Nayak, A., *Helicobacter pylori* in sewage Presented in 106th General Meeting of American Society for Microbiology. Orlando, FL. May 22-26 .2006.
  - Nayak, A., *Helicobacter pylori* qPCR Presented in 1st Annual Graduate Student Research Symposium Department of F&W, Michigan State University. East Lansing, MI. February 14th, 2006.
  - Nayak, A. *Helicobacter pylori* VBNC in sewage Presented in The 13th International Symposium on Health Related Water Microbiology Conference at Swansea , UK. Sept 4-9, 2005
  - Sangeetha Srinivasan, Shannon McGraw, Lauren Bull, Evangelyn Alocilja, Erin Dreelin & Joan B. Rose. Detection of waterborne pathogens using Real Time PCR and Biosensor methods. Presentation for the USEPA workshop on Innovative approaches for Detection of Microorganisms in water. Cincinnati, OH, June 18-20, 2007.
  - Sangeetha Srinivasan, Marc P. Verhougstreate & Joan B. Rose. Evaluation of Bacteroides, a new alternative indicator for fecal contamination. MI-ASM Branch Spring 2008 meeting at Central Michigan University, April 11-12, 2008.
  - Sangeetha Srinivasan & Joan B. Rose. New microbial source tracking methods for the water industry. Michigan Section, AWWA 70th Annual Conference. Kalamazoo, Michigan, September 9-12, 2008

Future Work

- qPCR:
  - Prepare a publication on the qPCR indicator studies.
  - Characterize the occurrence of 0157 in sewage and manure along with *E.coli* and Enterococci as indicators with qPCR.
- **Biosensor:**
  - Do test with seeded environmental water samples; do test using environmental water samples.
  - Test alterative design using SPCE biosensor.
- **Viability assay:**
  - Continue investigation into the replacement of currently employed biotinylated antibody with a more effective method of isolating *E. coli* from other bacterial contaminants prior to viability testing, in order to decrease cross-reactivity of developed assay.
  - Optimize the sensitivity and specificity determination of a strain-specific assay to detect viable *E. coli O157:H7* in surface water samples.
  - Implement alternative design using ATP-hexokinase system.
- **Biosensor-qPCR system**
  - Integrate biosensor-qPCR-viability assays into a seamless system.

Acknowledgment

- Funding sources for outputs of this project:
  - US Environmental Protection Agency
  - Department of Homeland Security through the National Center for Food Protection and Defense
  - Michigan Department of Environmental Quality
- Graduate students working on this project:
  - Shannon McGraw, Michelle Packard, Sangeetha Srinivasan
- Undergraduate students working on this project:
  - Lauren Bul, Teresa Brinks
- Postdoc working on this project:
  - Jongseol Yuk
- Other students who are members of the Alocilja Research Group


Thank You
Any Question?

Assessment of Microbial Pathogens in Drinking Water using Molecular Methods Coupled with Solid Phase Cytometry

Barry H. Pyle, Associate Research Professor
Department of Microbiology, Montana State University
U.S. Environmental Protection Agency Workshop on Innovative Approaches for Detecting Microorganisms and Cyanotoxins in Water, May 20-21 2009
Philadelphia, PA

COLLABORATORS

Anne Camper
Susan Broadaway
Al Parker
Jo-An Lindstrom
Montana State University
Bozeman, MT
Tim Ford
University of New England
Biddeford, ME

Overall Objective

- To develop and evaluate innovative approaches for quantitative assessment of pathogens

Target Microbial Pathogens

- *Escherichia coli* O157:H7
- *Helicobacter pylori*
- *Legionella pneumophila*
- *Mycobacterium avium*
- *Aeromonas hydrophila*
- *Giardia lamblia*
- *Cryptosporidium parvum*

Procedures

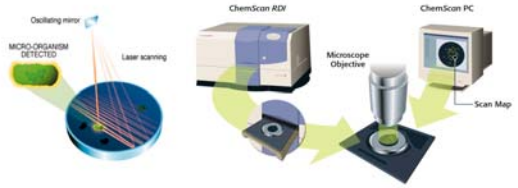
- Fluorescent *in situ* hybridization (FISH)
  - Enhance with tyramide amplification
  - Use polyamide nucleic acid (PNA) probes
- *In situ* nucleic acid amplification
  - Specific target genes inside individual cells (Hodson et al, 1995)
  - Improved methods, e.g. (Notomi et al, 2000; Maruyama et al, 2003 & 2005)
- Membrane filtration
- Solid Phase Laser Cytometry

Solid phase laser cytometry

- Scan a 25 mm diameter membrane filter in 3-4 minutes
- Detect individual fluorescent particles
- Discriminate between cells & debris
- Locate particles on microscope
- Validate bacteria, eliminate other particles

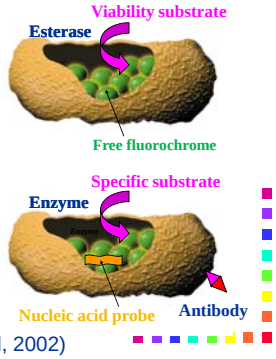
Solid Phase Laser Cytometer

ChemScan *RDI* (AES-Chemunex)

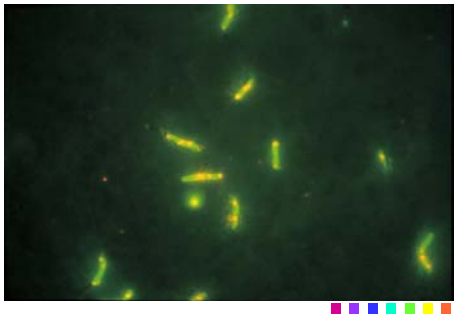


Range of Cell Labels

- **Total Cell Count**
  - Sybr Green
- **Total Viable Count**
  - ChemChrome
  - Enzyme activity
  - Membrane integrity
- **Identification Tests**
  - Antibodies
  - Specific enzymes
  - Nucleic acid probes
  - FISH
- **Dual Labeling**
  - Fab-CTC
  - ChemChrome-Fab
  - DVC-FISH (Baudart et al, 2002)

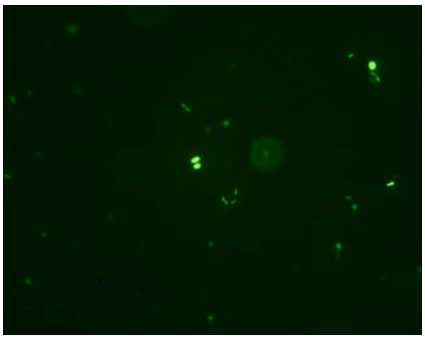


E. coli O157:H7, Immuno-magnetic Beads, CTC, FITC



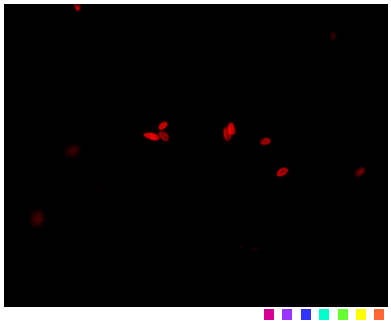
Pyle et al.,1999

CHEMCHROME V3-LABELED *Bacillus cereus*



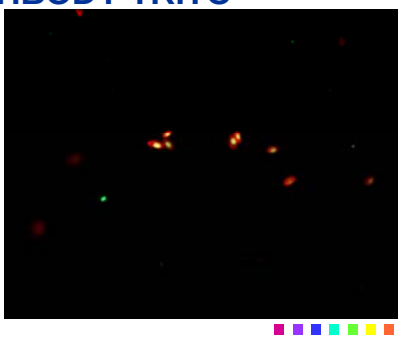
Pyle et al., 2000

B. cereus - B183 ANTIBODY WITH ANTI-MOUSE TRITC LABEL



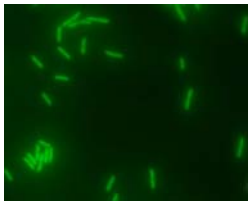
Pyle et al., 2000

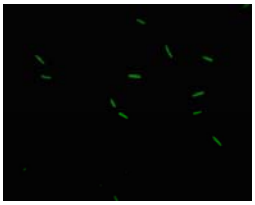
B. cereus - CHEMCHROME WITH B183 ANTIBODY-TRITC



Pyle et al., 2000

E. coli SYBR Green vs FISH





SYBR Green Stained

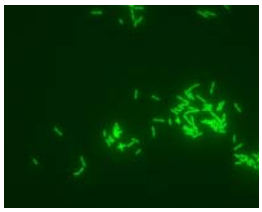
FISH with ECO-Alexa

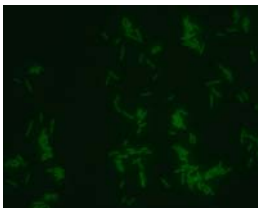
Images captured at same camera settings

Epifluorescent Microscopy

SYBR Green	FISH Eco Alexa	
Log CFU/ml	Log CFU/ml	
5.87	5.78	
6.19	6.38	
6.20	6.01	
6.09	6.06	Mean

SYBR Green vs FISH Tyramide





SYBR Green Stained

FISH-HRP with FITC Tyramide Amplification

Images captured at same camera settings

Goal Performance Characteristics

- Detection of different target bacteria with specific probes
- Detection of low numbers of pathogens
- Includes VBNC bacteria
- Can include infectivity and/or virulence
- Viable or active cells
- Single cell enumeration
- Sensitivity – 1 cell per filterable volume
- Rapid – Results within 6-8 hours

Scope of Project

- Drinking water and source waters
- Native American students at Little Big Horn College and Montana State University-Bozeman to participate

ACKNOWLEDGMENTS

- **U.S. Environmental Protection Agency**
  Barbara Klieforth, Project Officer
- **NIH Environmental Health Sciences**
- **NASA**
- **DoD – U.S. Army**
- **AES-Chemunex, Inc.**
- **LigoCyte Pharmaceuticals, Inc., Bozeman**
- **Montana State University**

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Maruyama, F., T. Kenzaka, N. Yamaguchi, K. Tani, and M. Nasu. 2003. Detection of bacteria carrying the stx2 gene by in situ loop-mediated isothermal amplification. Appl. Environ. Microbiol. **69**:5023-5028.

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Pyle, B.H., S.C. Broadaway, and G.A. McFeters. 1999. Sensitive detection of *Escherichia coli* O157:H7 in food and water by immunomagnetic separation and solid-phase laser cytometry. Appl. Environ. Microbiol. 65:1966-1972.

Pyle, B.H., S.C. Broadaway, J.T. Lisle, and G.A. McFeters. 2000. Improved detection of viable bacterial spores. Abstract Q-360, 100th Annual Meeting, American Society for Microbiology, Los Angeles, CA, May 21-25, 2000. (Poster). P. 624.

Detecting Pathogens in Water by Ultrafiltration and Microarray Analysis

Anthea K. Lee
Metropolitan Water District of Southern California

This research is funded by U.S. EPA - Science To Achieve Results (STAR) Program
Grant # R833004

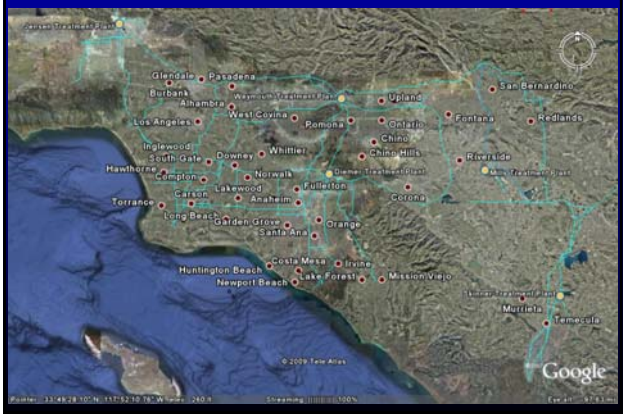
Metropolitan Water District of Southern California (MWD)

- Consortium of 26 cities and water districts
- Provide water for >18 million people in Southern California; 5200 square mile service area
- Delivers an average of 1.7 billion gallons of water daily

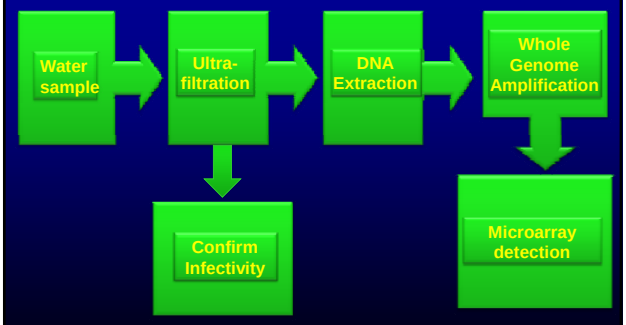
MWD Facts

- 5 treatment plants
- 5 pumping plants (1,617 feet lift total)
- 9 reservoirs
- 775 miles of pipeline

MWD System

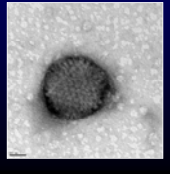


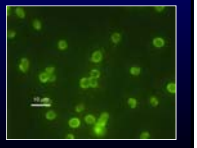
Pathogen Detection Strategy



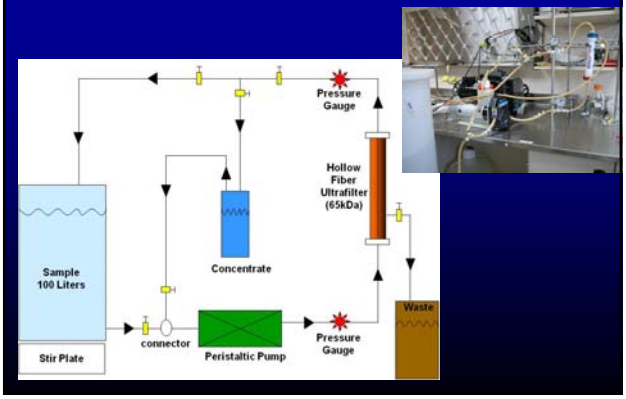
Model organisms

- *E .coli* K12
- *Salmonella typhimurium*
- *Cryptosporidium parvum*
- Human Adenovirus type 2

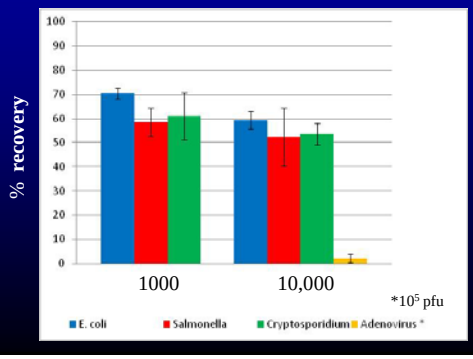




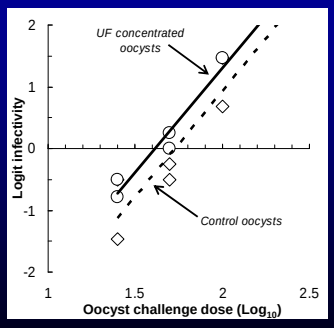
Ultrafiltration



Ultrafiltration Recovery

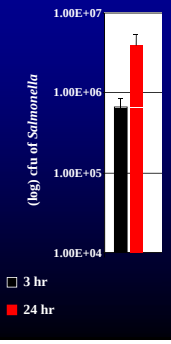


C. parvum survive ultrafiltration



ID_{50}

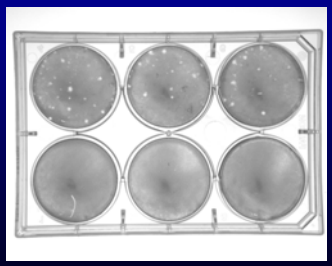
Salmonella Replication Assay



- Cell monolayer
- Infect with *Salmonella*
- Kill extracellular bacteria
- Incubate or not
- Lyse cells and plate intracellular bacteria

Adenovirus Plaque Assay

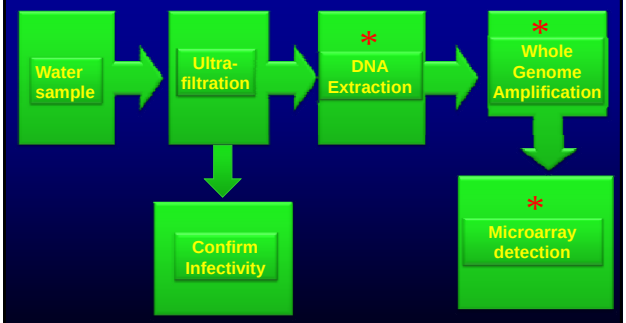
Infected



Uninfected control

- A549 human lung carcinoma cells
- Infect for 1 hour
- Add agar overly
- Incubate 7 days
- Stain with crystal violet
- Count plaques

Pathogen Detection Strategy



WGA for 10 ng starting material

Kit	expected yield* (ug/mL)	actual yield** (ug/mL)
REPLI-g Ultrafast Mini (Qiagen)	350-500	357 644
Illustra Genomiphi V2 (GE Healthcare)	200-350	317 214
GenomePlex Complete (Sigma)	40-93	30 none detected
DOP-PCR (Roche)	not specified	9 5

*Need 1-5 μg per microarray

WGA Results post-ultrafiltration

Organism (10^4 inoculum)	Extraction Kit	Mini WGA range (μg/ml)
E. coli	Invitrogen forensic kit	0.13-0.35
Salmonella	Invitrogen forensic kit	0.634-4.53
Cryptosporidium	MoBio ultraclean soil kit	1.95-8.39
Adenovirus	Invitrogen Purelink Viral RNA/DNA kit	Not done yet

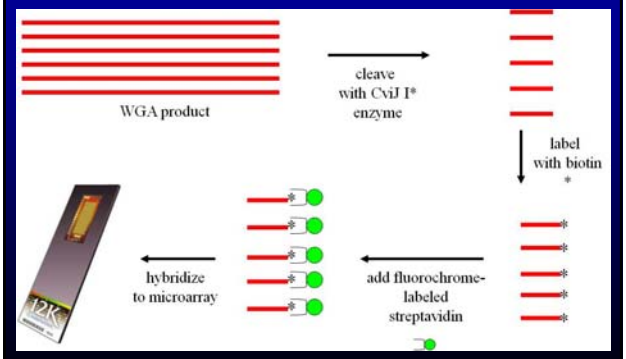
- 10^4 inoculum
- 17 fg DNA/bacterial cell
- Starting material ~0.01 ng DNA/10,000 cells
- Scaling up using Midi kit

E. coli K12 microarray to test integrity of WGA products

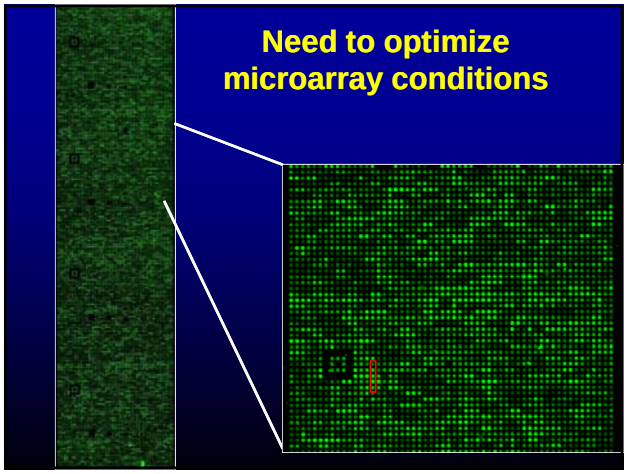
- 40 bp probes
- every 800 bp
- ~5800 probes
- cognate mismatch for each probe
- factory standard positive and negative controls

Combimatrix Custom Array

Target Preparation



Need to optimize microarray conditions



Summary of Progress

- Ultrafiltration recoveries for *E. coli*, *Salmonella* and *Cryptosporidium* are satisfactory
- Can use WGA to amplify genomic DNA recovered from ultrafiltration
- Infectivity confirmed for *Cryptosporidium*

Future Directions

- Optimize UF for Adenovirus
- Optimize larger scale WGA
- Optimize microarray parameters
- Finish infectivity studies
- Design custom microarray

Acknowledgments

Katrin Hanley
Jin Huang
Patty Huang
Anne Johnson
Paul Rochelle
Other colleagues at MWD

This research is funded by
U.S. EPA - Science To Achieve Results (STAR) Program
Grant # R833004

Robust PEMC Sensors for Detecting Pathogens in Drinking Water at 1 Cell/Liter

Raj Mutharasan
Sen Xu (PhD) Yanjung Ding (PDF)
Kishan Rijal (PhD), Gossett Campbell (PhD)
Department of Chemical and Biological Engineering
Drexel University

This research is funded by U.S.EPA - Science To Achieve Results (STAR) Program
Grant # R833007

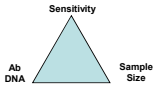
Innovative Approaches for Detecting Microorganisms in Water
Philadelphia, PA. May 20th, 2009

R833829

Research Objectives

1. Explore and establish experimentally piezoelectric-actuated millimeter-sized cantilever sensors suitable for detecting one pathogen in one liter of water using new cantilever oscillation and measurement modalities
2. Develop flow cell-PEMC sensor detection assembly for large sample volume
3. PEMC sensor for confirming pathogen identity by DNA signature

Motivation



- Waterborne parasites(*Cryptosporidium, Giardia) have low infective* dose (<10).
- Conventional methods require several days and need trained personnel. (EPA method 1622 and 1623)
- Large sample processing within a few hours without preparation is beneficial for environmental monitoring

Model parasites: *Cryptosporidium parvum* oocysts, *Giardia lamblia* cysts
Surrogates : *E. coli* O157:H7, *E. coli* JM101

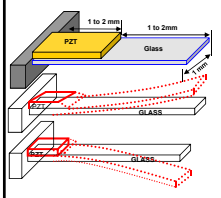
Progress

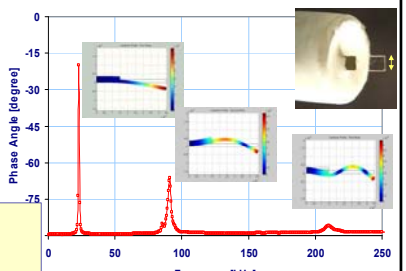
1. Sensitive mode established; flow cell (version 4 designed & tested) model experiments with *E. coli* O157:H7, Crypto and Giardia show detection limit ~ 10 – 50
2. Successful 1 liter samples completed using modified flow cell; 1 cell/mL completed
3. DNA-based detection of *E. coli* O157:H7 (*stx2* gene) at ~700 cells without amplification demonstrated in buffer

In Progress

1. Version-5 flow cell design and fabrication; river water Crypto at 10 and 100 liters
2. DNA-based detection of *Crypto* and *Giardia*

Piezoelectric-Excited Millimeter-sized Cantilever (PEMC) Sensors





Sensing Principle

- Resonant frequency depends on cantilever's mass.
- Surface is immobilized with a recognition molecule (eg. Antibody; ssDNA)
- When target attaches to the cantilever, mass changes, and resonant frequency changes

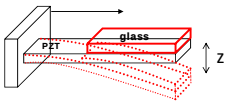
A virus weighs ~ 10^{-15} g (1 fg)
E. coli O157:H7 bacterium weighs ~ 10^{-12} g (1 pg)
A 30 kDa toxin weighs ~ $5x10^{-20}$ g (0.05 ag)
A 100-nt ssDNA weighs $5x10^{-20}$ g (0.05 ag)

5

Cantilever dynamics

Resonant frequency of Cantilever in air:

$$f_{nf} = k_n \sqrt{\frac{K}{M_e}} \quad (1)$$



PZT: Lead zirconate titanate ($Pb[Zr_xTi_{1-x}]O_3$ 0<x<1)

In liquid:

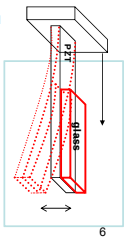
$$f_{nf} = k'_n \sqrt{\frac{K}{M_e + m_{ae}}} \quad (2)$$

Effective added oscillating liquid mass

When analyte of mass Δm binds:

$$f'_{nf} = k''_n \sqrt{\frac{K}{M_e + m_{ae} + \Delta m}} \quad (3)$$

stagnant or flow liquid



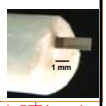
6

Dominant Higher modes= PEMC innovation

Higher frequency modes are more sensitive

$$f_n = \frac{\nu_n^2}{2\pi}\sqrt{\frac{EI/L^3}{\rho AL}} \Rightarrow \frac{\nu_n^2}{2\pi}\sqrt{\frac{k}{M}} \Rightarrow \frac{1}{2\pi}\sqrt{\frac{k}{(M/\nu_n^4)}}$$

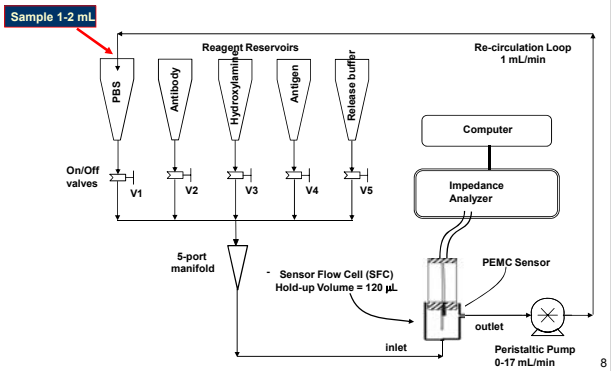
$$\text{Sensitivity} = \frac{\Delta m}{\Delta f_n} = \frac{k\nu_n^4}{2\pi^2}\frac{1}{f_n^3}$$



Only PZT is anchor

7

Experimental Apparatus



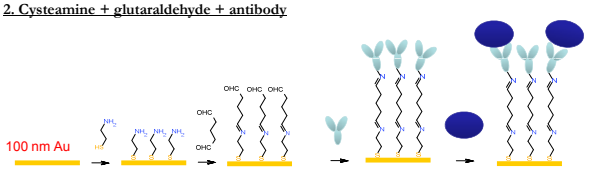
8

Interface Chemistry

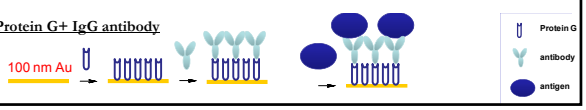
1. Amine-linked Immobilization

APTES (silane) → Glass → $-NH_2$ group → Ab+EDC/SulfoNHS → Covalently immobilized Ab

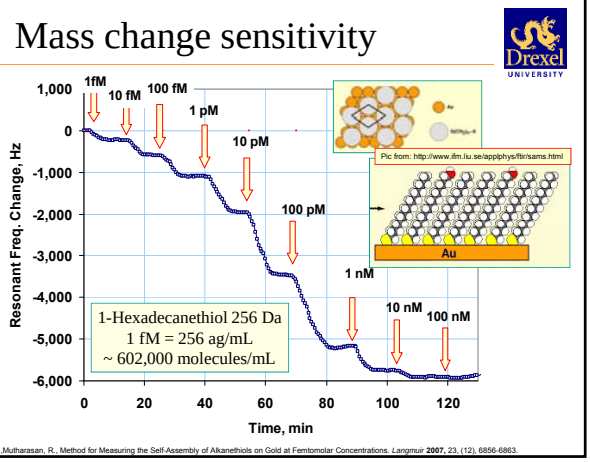
2. Cysteamine + glutaraldehyde + antibody



3. Protein G+ IgG antibody

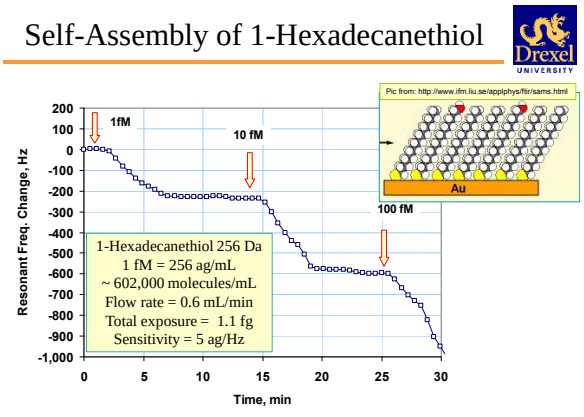


Mass change sensitivity



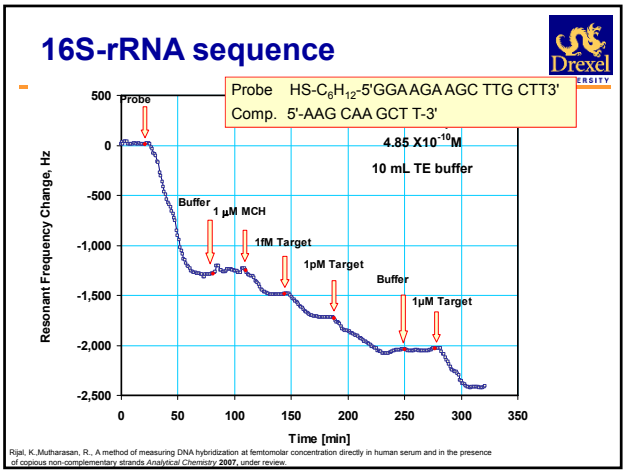
Rijal, K.,Mutharasan, R., Method for Measuring the Self-Assembly of Alkanethiols on Gold at Femtomolar Concentrations. *Langmuir* **2007,** 23, (12), 6856-6863.

Self-Assembly of 1-Hexadecanethiol



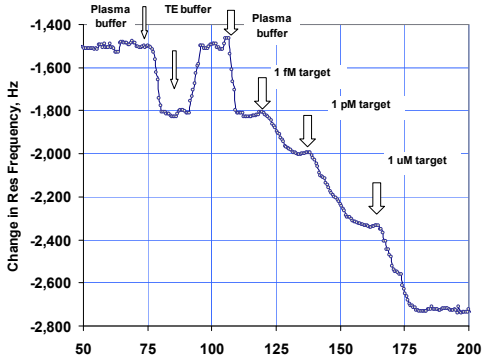
Rijal, K.,Mutharasan, R., Method for Measuring the Self-Assembly of Alkanethiols on Gold at Femtomolar Concentrations. *Langmuir* **2007,** 23, (12), 6856-6863.

16S-rRNA sequence



Rijal, K.,Mutharasan, R., A method of measuring DNA hybridization at femtomolar concentration directly in human serum and in the presence of copious non-complementary strands *Analytical Chemistry* **2007,** under review.

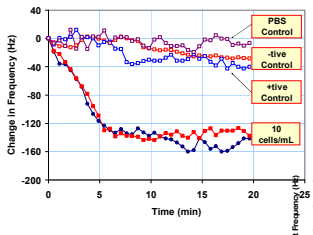
16S-rRNA - human serum

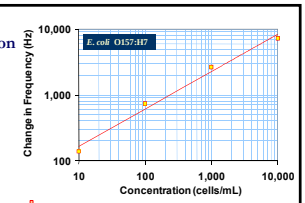


Rijal, K.,Mutharasan, R., A method of measuring DNA hybridization at femtomolar concentration directly in human serum and in the presence of copious non-complementary strands *Analytical Chemistry* **2007**, under review.

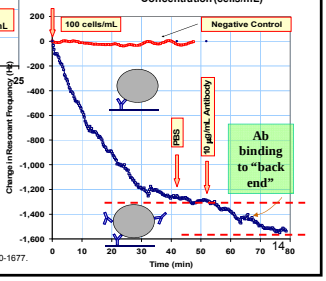
E. coli O157:H7 – Confirmation, Repeatability and quantitation

Au-Protein G-Ab



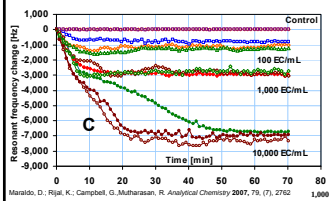


- control: *E. coli* absent, Ab present
+ control: *E. coli* present, Ab absent



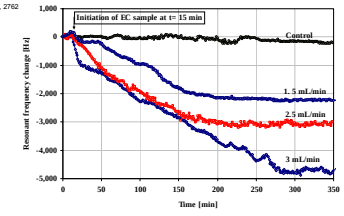
Maraldo, D. and Mutharasan, R. *Journal of Food Protection* **2007**, 70(7), 1670-1677.

E. coli O157:H7 in water [APTES-Immobilization]



Maraldo, D.; Rijal, K.; Campbell, G.,Mutharasan, R. *Analytical Chemistry* **2007**, 79, (7), 2762

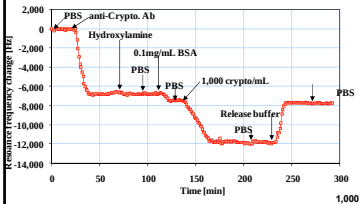
Repeatability



One Liter Sample

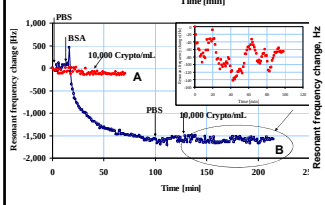
Campbell, G. A.,Mutharasan, R.*Environmental Science Technology* **2007**, 41, (5), 1668-1674.

Cryptospordium oocyst in tap water

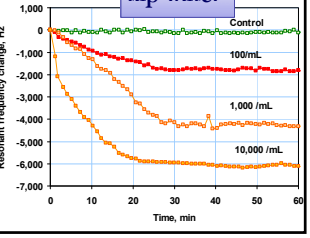


IgM immobilized via APTES

Sensor response is proportional to concentration



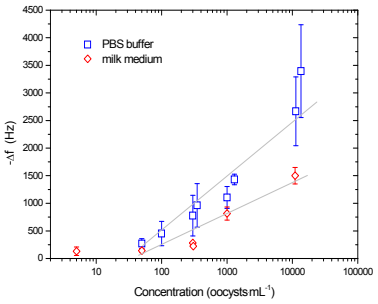
tap water



Campbell and Mutharasan *Biosensors and Bioelectronics* **2008**, 23(7):1039-10

Quantification of *C. parvum* in milk and in PBS

Protein G + Goat IgG



$$(-\Delta f) = A\log(C_{Crypto}) + B$$

- Both in PBS and in milk gave good semi-log correlation.
- Detection in milk is 40-50% less sensitive than in PBS

S. Xu and R. Mutharasan, manuscript in preparation

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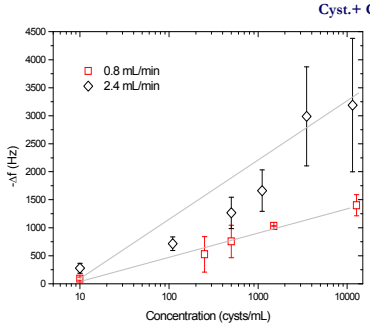
Flow rate influences sensor response

Cyst.+ Glutaraldehyde + mono IgG



$$(-\Delta f) = A\log(C_{Giardia}) + B$$

- Different sensors with variations (n=11) gave good correlation
- High flow rate gave larger response

S. Xu and R. Mutharasan, manuscript in preparation

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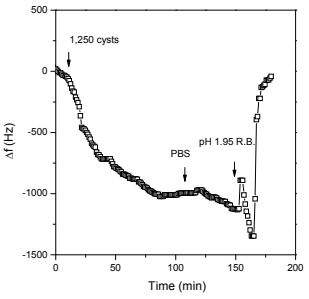
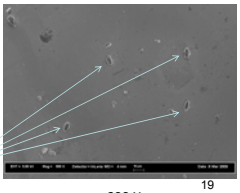
Confirmations w/ release & SEM imaging



6,000 X



600 X

G. lamblia cysts

Cyst.+ Glutaraldehyde + mono IgG

Sample preparation

Probe: HS-C_6H_{12}-5'-CCA CTC TGA CAC CAT CCT C-3'
One genomic copy per cell
Stx2 codes for shiga-like toxin2. Probe design - Primer 3

1.5 mL PBS + 50,000 *E. coli* O157:H7 or JM101

30-min prep

- Centrifuge at 10,000 g for 5 min
- Pellet + 50 µL 1% Triton X-100
- 10 mins in boiling water, cool at 2-3 °C for 15 mins
- Centrifuge at 10,000 g for 3 mins
  Dilute supernatant in 10 mL TE buffer
- 1 mL- Shear 25X with 30-gauge 1/2" needle
  23 pg/mL genomic DNA

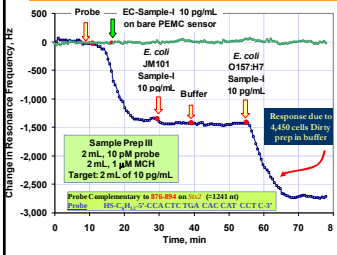
Fermentas Genomic DNA extraction kit

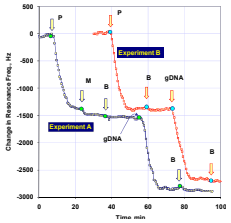
- Stock 1.5 ng/mL genomic DNA (measured)
- Diluted to 23 pg/mL

DNA-based Detection [2 mL at 10 pg/mL genomic DNA]

Rijal and Mutharasan, unpublished

Stx2-probe based detection– Prep I (buffer)



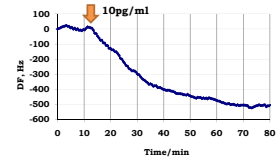


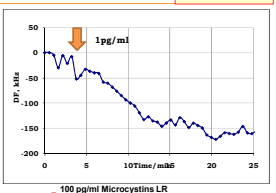
Samples	gDNA (pg/mL)	Sensor Response to Hybridization (Hz)	Hybridization rate (min^{-1})
Sample-I Extraction from buffer [with MCH]	10	-1,255±19	0.18
Sample-III Extraction & purification using a commercial kit	10	-1,302±21	0.21

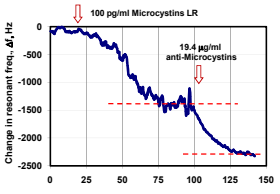
Rijal and Mutharasan, 2009 TBS

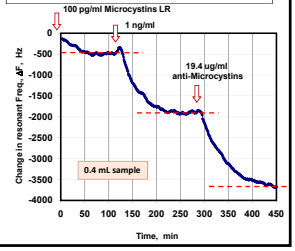
Detection of Microcystin LR in batch mode

R833829









Yanjun Ding and Mutharasan, unpublished

Conclusions

- **Cantilever sensor mass change sensitivity = 1 ag/Hz**
- ***E. coli* in buffer – Detection limit – 10 (in theory one cell)**
- **One liter sample detection shown**
- **Crypto and Giardia in buffer & proteinous environment – ~ 10**
- **Stx2-gene based detection –– ~700 cell detection. 100 appears to be feasible**

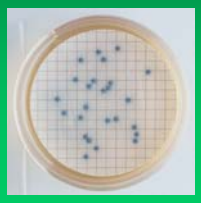
MST research

Jorge Santo Domingo
US EPA
NRMRL/WSWRD/MCCB
Cincinnati, OH

Monitoring Fecal Pollution

Microbial "Fecal Indicators"

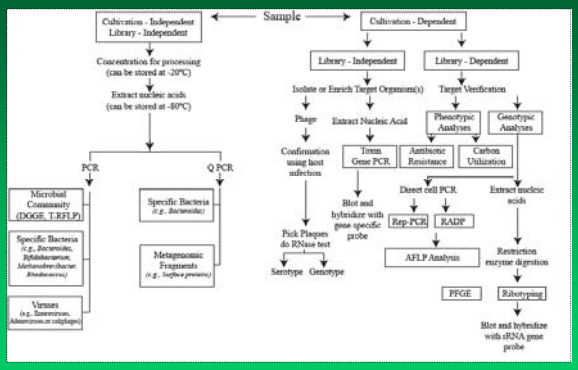
- **Indicate presence of fecal pollution and potential health risks, not when it happened or what is the source**



Microbial Source Tracking or
Fecal Microbial Forensics

Use of detectable molecular variations between related fecal microbial strains to infer the origin of pollution sources in a fecally contaminated watershed
(or food supply).

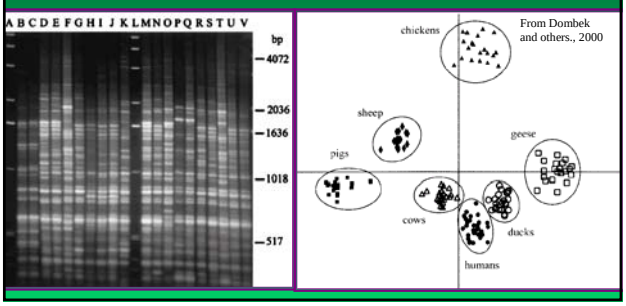
Many methods and more to come!!!



Adapted from Cindy Nakatsu; EPA's MST guide

Library-dependent methods

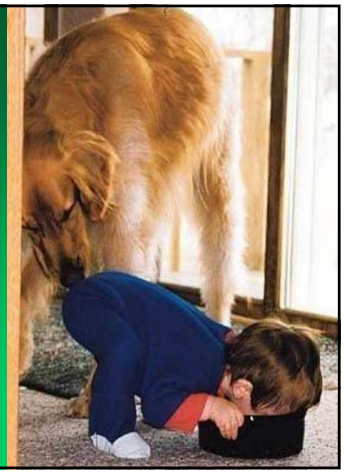
From this ... to this



NOT

Source Trackers

BEST FRIEND!!!

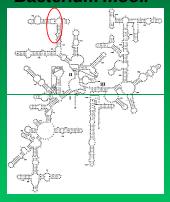


Host-Specific PCR Assays

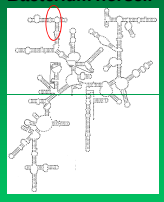
- Culture-independent
- Library-independent
- Rapid detection
- Sensitive
- Defined target
- Automated analysis
- Potential for multiple assays
- Potential for really cheap assays

Comparative Sequence Analysis of 16S rDNA

16S rRNA of *Bacterium mooii*



16S rRNA of *Bacterium horseii*



	650	660	670
B. mooii	GCUUGAGUCU	CGUAGAGGGG	GGUAGAAUUC
B. horseii	GCUAGAGUAU	GGGAGAGGAU	GGUAGAAUUC

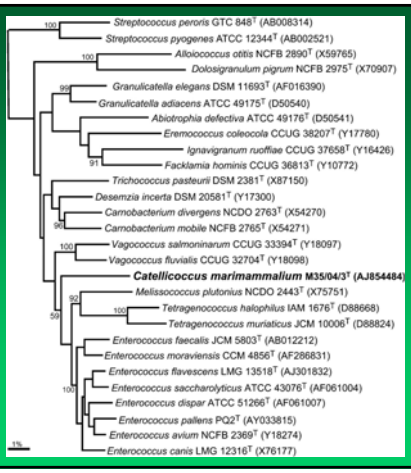
Steps for assay development

- DNA extract from feces
- PCR amplification w/ 16 rDNA primers
- Cloning, sequencing, blast, and phylogenetic analysis
- Rare groups used for assay development

Development of gull assays

Phylogenetic affiliation of 16S rRNA genes from gull fecal clone library

Class or group (% clones of total)	Genus	No. of clones
Actinobacteria (6.4)	*Corynebacterium*	8/18
Bacilli (37.2)	***Catellicoccus***	**74/105**
Bacteroidetes (1.1)	*Bacteroidetes*	1/3
Clostridia (17.31)	*Clostridium*	44/49
Fusobacteria (0.7)	*Cetobacterium*	2
Mollicutes (8.8)	Unknown genus	25
Alpha proteobacteria (6.7)	*Paracoccus*	8/19
Beta proteobacteria (4.3)	*Acidovorax*	6/12
Gamma proteobacteria (11.3)	*Acinetobacter*	13/32
Delta proteobacteria (0.4)	Unknown genus	1/1
Epsilon proteobacteria (0.4)	*Campylobacter*	1/1
Planctomycetes (0.4)	*planctomycete*	1/1
Spirochaetes (1.1)	*Leptospira*	3/3
Cyanobacteria (0.4)	*Synechococcus*	1/1
Archaeon (0.4)	Unknown genus	1/1
Unknown Class (3.2)	Unknown genus	9/9



Host-specificity tests of gull assay (Gull2) against feces from various animals

Animal	Locations of samples	Samples tested	Gull2 Normal PCR	Real-Time C_T
Pig	DE	10	-	BDL
Cow	WV	28	-	BDL
Human	WV	16	-	BDL
Goat	DE	10	-	BDL
Sheep	DE	11	-	BDL
Horse	WV	5	-	BDL
House cat	WV	11	-	BDL
Domestic Dog	WV	13	-	BDL
Coyote	TX	10	-	BDL
Deer	WV	6	-	BDL
California Sea lion	CA	24	-	ND
Black Vulture	TX	1	-	BDL
Canadian goose	WV	62	-	BDL
Turkey	DE	19	-	BDL
Pigeon	WV	5	-	BDL
Duck	GA	25	-	BDL
Chicken	WV	14	-	BDL
Penguin	OH	3	-	BDL
Parrot	OH	4	-	BDL
Dove	OH	2	-	BDL
Pelican	OH	1	-	BDL
Ibis	OH	1	-	BDL
Gull	WV	8	+	23.41

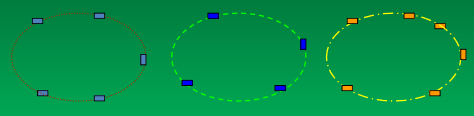
Host distribution of gull fecal-specific PCR assays and average 16S rRNA gene copy numbers

Target	Sampling locations	Numbers of fecal samples	Gull2		
			PCR – No. of positive samples	qPCR	
				No. of positive samples	Average copy no. per ng DNA ± std dev
Larus delawarensis	GA	13	10	10	6117±12428
Larus atricilla	GA	20	10	12	905±1040
Larus atricilla	OH	3	3	3	414±496
Larus delawarensis	OH	3	2	3	52±73
Larus delawarensis	WV	8	7	6	896±932
Larus atricilla	FL	7	5	5	216±171
Larus delawarensis	Ontario, Canada	4	4	4	93044±71792

Detection of gull feces in environmental water samples using gull-specific (Gull2) assay

Sampling locations	Sample type	Time of collection	Water samples	No. of positive samples
Grant Park Beach, WI (Lake Michigan)	Freshwater	September-October, 2007	8	8
Maumee Bay, Oregon, OH (Lake Erie, OH)	Freshwater	October, 2007	3	3
Toledo Botanical Garden Pond (Toledo, OH)	Freshwater	October, 2007	2	0
Northeast, OH	Chicken pit	2007	9	0
Northeast, OH	Pig pit	2008	3	0
Northeast, OH	Cow manure lagoon	2008	1	0
Southern, GA	Fresh water	2007	9	0
Bayfront Park Beach (Lake Ontario, Canada)	Freshwater	May-August, 2007	10	10
Bluffers Park Beach (Lake Ontario, Canada)	Freshwater	May-August, 2007	10	10
Sunnyside Beach (Lake Ontario, Canada)	Freshwater	May-August, 2007	10	10
Doheny State Beach Pond (Dana Point, CA)	Freshwater	June-July, 2007	7	7

Searching for Novel Chicken MST Markers

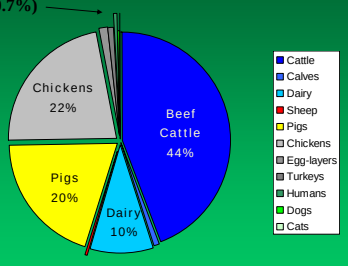


Multiple genes – Multiple bacterial groups

Unlike 16S rDNA, little sequence data is available for host specific functional genes

Feces productions in the U.S.

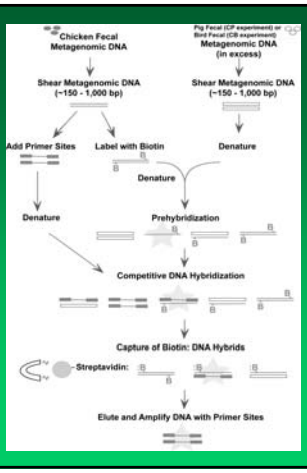
Human (0.7%)



Plus Wildlife!

- $1x10^{12}$ kg/year

Genome
Fragment
Enrichment



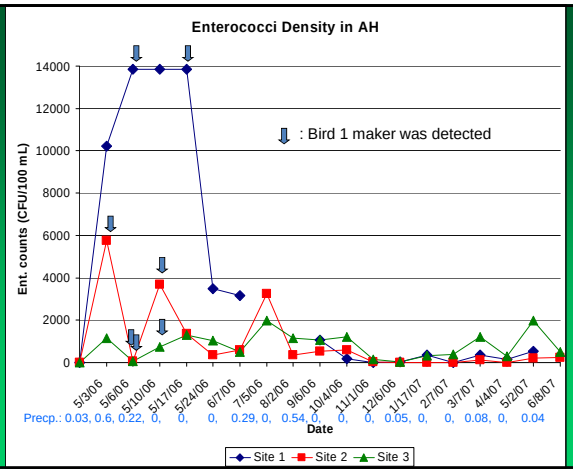
Lu et al. Water Research 2007

Next steps

- Fragments are cloned and sequenced
- Sequences are classified by function and potential bacterial host
- Sequences associated with host-microbial interactions are used to develop PCR assays
- Assays are tested for host-specificity, host-distribution, and detection limits (both in fecal sources and water samples)

Description of primers tested for host specificity

Clone #	Fragment Size/PCR product size (DNA bp)	Primer set and sequences (5'→3')	COG category	Top BLASTX hit organism	Expect values	Amino acid length of match for BLASTX alignment (% identity)	Primer specificity
CB-R2-10	326/306	CCATCCACAGCACGTCGTA AGATCTTCATCCAGTACGGCA	Cellular processes (chaperones)	*B. fragilis*	4E-27	108 (50%)	Chicken & Goat
CB-R2-27	614/607	CGAAGCGGAGAAGAACAAGA GTTCCGCAACGTAGAGGAAA	Metabolism (Inorganic ion)	*B. thetaiotaomicron*	2E-44	205 (45%)	Chicken, goat & sheep
CB-R2-28	344/327	GGCAAGCCTCAATCGCAT GTTCTGGTCGTTGGGCTGA	Cellular processes (Signal transduction)	*B. fragilis*	3E-35	115 (61%)	Chicken & sheep
CB-R2-34	418/261	CTCCAGGATTTCGTGGGA AAGGAGCAGCTGACGGCA	Information storage and processing	*Clostridium thermocellum*	5E-26	115 (52%)	Chicken, pigeon & Sheep
CB-R2-42	627/265	GACGAGATCTATATTTGCCTCA CGGAGCATATCTCTACGATCA	General function prediction only	*Desulfitobacterium hafniense*	1E-03	93 (33%)	Chicken
CB-R2-80	589/287	CGTGAATTTCCGCTACGA CCTCTTCCTTGCGTCCCA	Cellular processes (wall/membrane)	*B. fragilis*	1E-25	125 (45%)	Chicken
CP1-1	623/281	GGCAGGCATCAAGTCAACA TGGCAAAGCAACTGTCATGGCA	Cellular processes (cell division)	*C. tetani*	3E-16	99 (41%)	Chicken & other birds
CP-1-10	383/350	AGGAGCATTTGTCGCCCTA GGTAAGCTGCCCGGTAATA	Cellular processes (defense)	*B. fragilis*	9E-31	96 (88%)	Chicken
CP1-24	549/379	TACCCGCAACGGGGAGAA CCGATGATACGCTTTCCAA	Metabolism (Inorganic ion)	*B. fragilis*	3E-13	138 (33%)	Chicken
CP1-25	575/445	CTGGAGATCATCGTTGACAGA TAGGCTCAAGCAGTACCGGA	Information storage and processing	*C. perfringens str.*	4E-58	165 (65%)	Chicken & turkey
CP1-26	544/442	CCTGTCGTAAAACCCGGGG TCTTCGATTTTCCCTGTTTCA	Metabolism (Carbohydrate)	*B. thetaiotaomicron*	3E-37	162 (44%)	Chicken
CP1-40	438/244	TATTTCTGGGTGCGGTTGTA CTGACCGGAATGACTCCCA	General function prediction only	*B. thetaiotaomicron*	6E-6	114 (30%)	Chicken



Lessons learned

- Detection limits can vary dramatically per fecal sample, host, water sample
- Host distribution can also vary considerably
- Preferential distribution and secondary habitats issues like *E. coli*
- Different markers for different sources of the same fecal sources
- Combination of assays best approach to enhance confidence levels

Lessons learned

- The more (markers) the merrier; you never know which marker will work
- Survival of the targeted population is rather important
- Feces might not always be the best starting point for assay development
- There is unknown bacterial groups that might be used for assay development
- Abundance of host-specific populations can vary

Regional projects

RARE Project – Evaluate MST assays in tropical inland waters

Regional Methods Program – Comparison of MST and PST assays

Acknowledgements

USEPA Computational Toxicology Grants
USEPA WSWRD

Jingrang Lu – NRC Award
Regina Lamendella, Daniel Oerther – UC
Rod Mackie, Tony Yanarell – UIUC
George DiGiovanni – UT El Paso
Stephen Hill, Tom Edge – Environment Canada

Rapid Concentration, Detection, and Quantification of Pathogens in Drinking Water

Zhiqiang Hu, Department of Civil and Environmental Engineering
Lela K. Riley, Department of Veterinary Pathology
Mengshi Lin, Department of Food Systems & Bioengineering
University of Missouri, Columbia MO 65211

Outline

- **Lanthanum-Based Concentration and Microrespirometric Detection of Microbes in Water**
  - Turbidity-based and Fluorescence-based microrespirometry to enumerate microbes and determine microbial activity in water
  - Lanthanum-based microbial concentration
- **Rapid detection and quantification of water-borne pathogens by SERS coupled with nanosubstrates**

Part I. Lanthanum-Based Concentration and Detection

Introduction

- Rapid detection of potential pathogens in water is crucial to drinking water supplies.
  - The numbers of microorganisms in water samples are often too low to be detected.
- Coagulation/flocculation coupled with filtration is an attractive method for concentration.
- $LaCl_3$ is a flocculant that can concentrate microbes by strong electrostatic interaction.
  - Compared with traditional flocculants (e.g., alum and ferric salts), $LaCl_3$ only hydrolyzes slightly in the water so that it minimize the impact on microbial properties.

Introduction

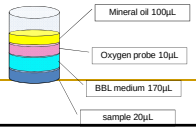
- Traditional assays enumerate microbes by measuring the turbidity of the organisms.
- Oxygen-based microrespirometry, however, can enumerate the live microbes by measuring oxygen consumption and determine microbial activities at the same time.
- Lanthanum chloride was used to concentrate the microbes in water before they were detected and quantified by microrespirometry.

Materials and Methods

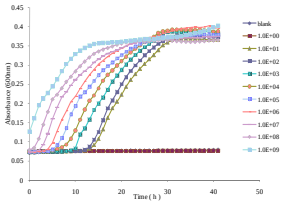
- Bacterial Strain used: *E. coli* (ATCC 47076)
- Flocculants/Coagulants: $LaCl_3$, $FeCl_3$ and $Al_2(SO_4)_3$ (final concentrations = 0.2 mM).
- Concentration procedures
  - Mixed at 200 rpm for 1 min, followed by slowly mixing at 30 rpm for 20 min.
  - The samples were allowed to settle for 1 hour.
  - The supernatant fluids (75mL) were carefully removed without disturbing the flocs.

Microrespirometric Detection

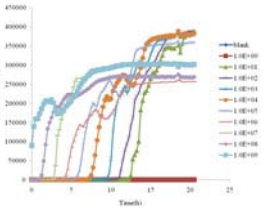
- Composition in microwells
  - For every flocculant treatment, aliquots (20 µL) of supernatant or sediment samples were taken and added to the microplate wells followed by the addition of 180µL BBL medium.
- Turbidimetric assay
  - The microtiter plate was read at 600 nm.
- Microrespirometric dectection
  - Oxygen probe and mineral oil were added.
  - Time-resolved fluorescence measurements were recorded with 340 nm excitation and 642 nm emission



Time profiles of *E.coli* growth at different initial cell concentrations

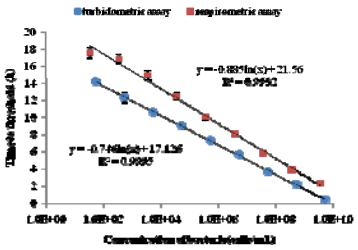


Turbidimetric assay

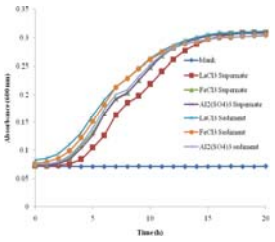


Microrespirometric assay

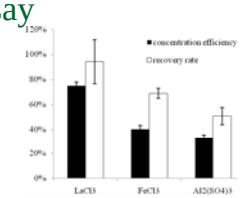
Strong Correlation between Bacterial Concentration and Time to Threshold



Concentration study Using turbidimetric assay

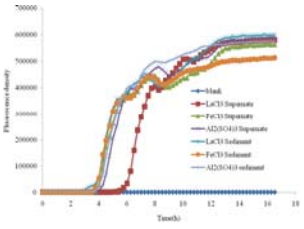


Time profiles of absorbance of different samples

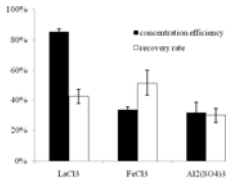


Concentration efficiencies and recovery rates of different treatments

Concentration Study based on microrespirometric assay

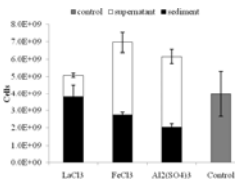


Time profiles of oxygen probes signals of different samples

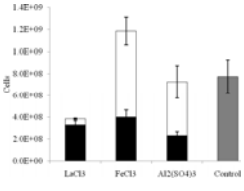


Concentration efficiencies and recovery rates of different treatments

Bacterial Distribution Using Different Flocculants

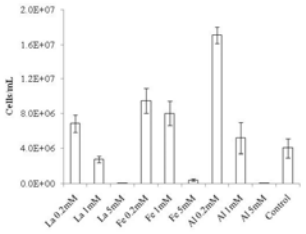


Turbidimetric assay

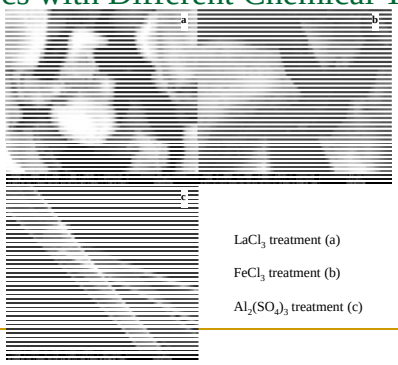


Microrespirometric assay

Effect of flocculants ($LaCl_3$, $FeCl_3$ and $Al_2(SO_4)_3$) on *E. coli* bacterial growth



Microscopic (ESEM) Examination of Flocs with Different Chemical Treatment



$LaCl_3$ treatment (a)

$FeCl_3$ treatment (b)

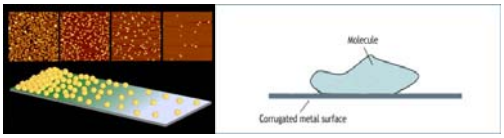
$Al_2(SO_4)_3$ treatment (c)

Summary

- Compared with traditional flocculants, $LaCl_3$ has the highest relative concentration and recovery efficiencies. The lanthanum-based method coupled with ultrafiltration provides a promising pathogen concentration method for water utilities.

Part II. Rapid detection and quantification of water-borne pathogens by SERS coupled with nanosubstrates

Surface enhanced Raman spectroscopy (SERS)

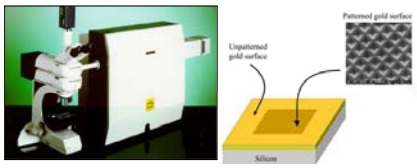


- When analyte molecules are adsorbed on metal surface with nanoscale roughness, Raman signal can be tremendously enhanced due to spatially localized surface plasmon resonance (SPR) from the "hot spots" where huge local enhancements of electromagnetic field are obtained.
- The enhancement factor can be more than 10^6. Limit of detection can reach the parts per billion (ppb) level or possibly a single molecule

(www.innovations-report.com; D3 Technology)

Introduction

SERS and nanosubstrates

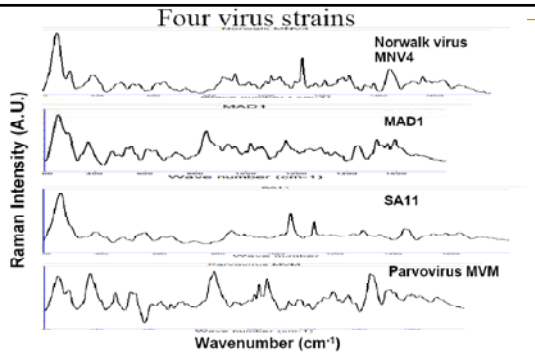


Renishaw RM 1000 Klarite™ substrates

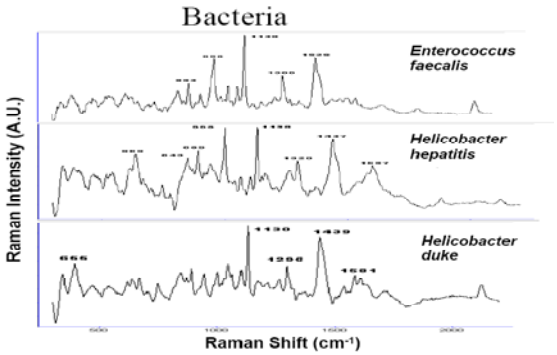
A Renishaw RM1000 Raman spectrometer system with 785 nm near-infrared diode laser source;
Gold substrate (Klarite): fabricated on silicon wafers coated with gold, nanotextured pyramidal subunits.

Objective

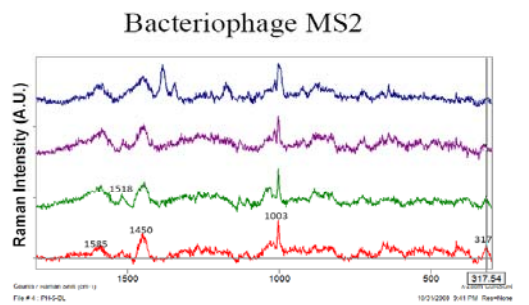
- To develop and validate SERS-based method for pathogen detection and quantification.
- Several species representing the major categories of pathogens in drinking water were chosen for SERS testing:
  - *Enterococcus faecalis*
  - *Helicobacter pylori*
  - Human adenovirus
  - Calicivirus
  - *Encephalitozoon cuniculi*
  - *E. coli* O157:H7
  - *Cryptosporidium parvum*



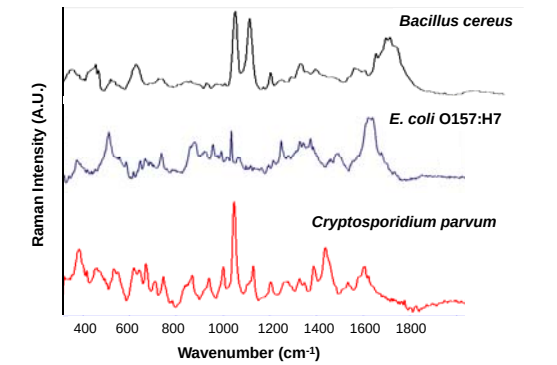
- SERS spectra of four virus strains show "fingerprint-like" spectral patterns that can be used to classify and identify these strains; gold nanosubstrates were used in measurement.



- Distinctive SERS spectral patterns were observed between three waterborne bacteria

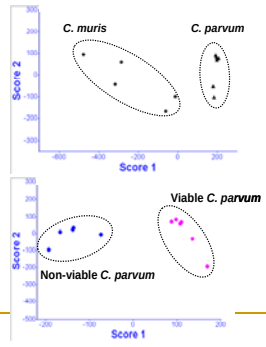


- We also collected SERS spectra of bacteriophage MS2 on gold nanosubstrates



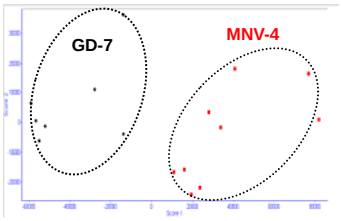
- Three bacterial pathogens exhibit different SERS spectra that can be used to identify them

Principle Component Analysis (PCA) of *Cryptosporidium* spp.



- Clear data segregations were obtained between *C. muris* and *C. parvum*; and viable and non-viable *C. parvum*.
- These results indicate that SERS can be used to identity and discriminate between different *Cryptosporidium* oocysts as well as viable or not based on their unique and distinct vibrational spectral information.

PCA of GD-7 (Picornavirus) and MNV-4 (Norovirus)



- **PCA was able to classify two virus stains:** GD-7 (Picornavirus) and MNV-4 (Norovirus)

Band assignment of Raman peaks in the range of 300 – 2200 cm^{-1}

Raman Shift (cm^{-1})	Assignment
~ 540	COC glycosidic ring def
~ 620	Amino acids (Phe)
~ 640	Nucleic acids (T)
~ 665	Nucleic acids (G)
~ 726	Nucleic acids (A)
~ 783	Nucleic acids (C, T)
~ 853	Tyrosine
~ 936	DNA backbone
~ 977	Lipid (C=C deformation)
~ 1005	Phenylalanine
~ 1035	Carbohydrates (C-C deformation)
~ 1101	DNA (O-P-O stretching)
~ 1128	C-N stretching
~ 1252	Amide III
~ 1340	Nucleic acids (A, G)
~ 1453	Lipid ($C-H_2$ deformation)
~ 1577	Nucleic acids (A, G)
~ 1665	Amide I
~ 1735	>C=0 ester str

(Maquelin and others 2002)

Summary

SERS coupled with nanosubstrates and statistical tools shows great potential to rapidly detect and identify different water-borne pathogens.

This research is funded by U.S. EPA - Science To Achieve Results (STAR) Program
Grant # 83384001

Acknowledgements

Funded by EPA STAR Program (#83384001)

The effect of flocculation on pH

Before flocculation	7.01		
After flocculation	La^{3+}	Fe^{3+}	Al^{3+}
0.2mM	7.03	7.00	7.05
1mM	6.62	6.54	6.63
5mM	5.43	2.44	4.19

Simultaneous Concentration and Real-time Detection of Multiple Classes of Microbial Pathogens from Drinking Water

Prof. Mark D. Sobsey
Department of Environmental Sciences and Engineering
Gillings School of Global Public Health
University of North Carolina
Chapel Hill, NC 27599-7341

Objective 1

- Refine and validate new and improved, rapid **hollow fiber ultrafiltration methods** to concentrate viruses and cellular pathogens (bacteria and protozoan parasites) from waters of variable quality
  - Particles
  - Dissolved organic matter
- Compare to existing virus concentration methods (1MDS VIRADEL)

Objective 2

- Fabricate (or identify) and evaluate improved and cost-effective **electropositive filters** to rapidly and efficiently concentrate enteric viruses from waters of different quality by adsorption to and elution
  - Nanoceram cartridge filter (Argonide)
- Compare to existing virus concentration methods (1MDS VIRADEL)

Objective 3

- Improve and evaluate post primary concentration sample preparation techniques:
  - Rapid **PEG precipitation**
  - Post PEG precipitation treatments to improve virus detection by quantitative real-time (RT-)PCR
  - Large volume **nucleic acid extraction**
- Further concentrate viruses
- Remove inhibitors
- Facilitate efficient, specific, and sensitive real-time, molecular detection of viral nucleic acids
  - Human adenoviruses
  - Human enteroviruses
  - Human noroviruses

Objective 4

- Improve and optimize direct detection of viral RNA/DNA by real-time molecular methods for rapid and efficient detection of low numbers of target viruses
  - Sample volume per (RT-)PCR reaction
  - Additives to (RY-)PCR mixtures

Objective 5

- develop complete protocols of the methods and provide them to a select number of other water virology laboratories to conduct a collaborative (round-robin) test of the methods that characterizes their performance; and

Concentration of Adenoviruses, Noroviruses and Echoviruses from Water

- Primary concentration
  - Recirulating flow hollow fiber ultrafiltration
    - 2 brands of filters
    - Modified endcaps to increase flow rate/flux
    - Alternative beef extract elution solutions
    - Performance in waters of different quality (source and treated)
  - Once-through, gravity-flow hollow fiber ultrafiltration
  - Nanoceram electropositive adsorbent filter
    - Nano alumina (AlOOH) fibers
    - Virus concentration from seawater
- Secondary concentration
  - Polyethylene glycol precipitation
    - Effect of PEG and NaCl concentrations

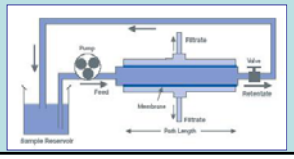
Recirculating HFUF Methods and Materials

Hollow-fiber ultrafilters (HFUF):

- Fresenius F80A
  - (Fresenius Medical Care, Lexington, MA)
- Hemocor HPH
  - (Minntech Corporation, Minneapolis, MN)

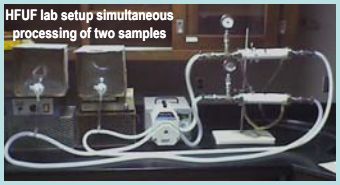
HFUF flow modifications:

- Modified end caps with larger diameter openings
- Increased flux for more rapid sample processing



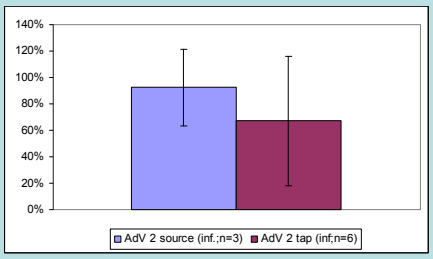
Recirculating HFUF Methods and Materials

- Test water: ≥10-liter volumes of untreated source and de-chlorinated finished waters (SFPUC: San Francisco Public Utility Commission)
- HFUF units: ca. 75,000 MWCO, designed for kidney dialysis
- Peristaltic (flexible tubing roller) pump to re-circulate water through the unit
- As water re-circulates, permeate is separated from retained particles, concentrating particles, including microorganisms, to <300 ml volume

Recovery of Adenovirus 2

- Hollow Fiber Ultrafiltration
  - Virus assay by cell culture infectivity



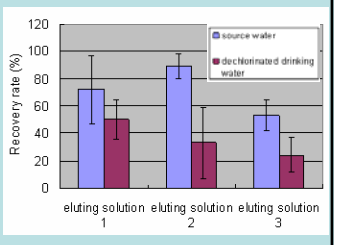
HFUF Recovery of Adenovirus 41

- Eluting solution comparison for Ad41 recovery from HFUF primary concentrates

Eluting Solution 1 (Standard)
1 L Phosphate-buffered Saline (PBS)
10 g laureth-12
50 µL antifoam-A

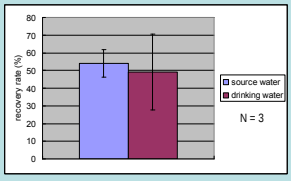
Eluting Solution 2
1 L PBS
10 g laureth-12
1 g NaPP
50 µL antifoam-A

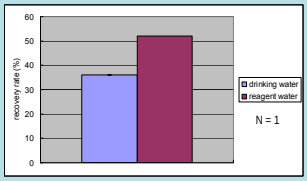
Eluting Solution 3
1 L reagent water
52.7 g L-Arginine (A-5131) (0.25 M)
45.65g L-Lysine (L-5826) (0.25 M)
10 g laureth-12
50 µL antifoam-A



HFUF Recovery of Adenovirus 41

- Lower spike virus concentration (10^5/10L) (Left)
- Recovery from large volume (100L) (Right)





HFUF Recovery of Pathogenic Microbe Suite

Organism	Spike Conc'n. (cfu,pfu/L)	Source Water		Drinking Water	
		Trials (N)	Average Recov. (%)	Trials (N)	Average Recov. (%)
E. coli O157	500	3	52±6	3	44±12
Salmonella	500	3	85±13	3	117±27
Aeromonas	500	3	11±3	3	7±5
Echovirus-12	2000	3	49±45	3	ND
Cryptosporidium	20	3	29±11	3	28±6
Giardia	20	3	9±3	3	15±8

ND = No Data (eluting solution 2 with NaPP was toxic to cell cultures)

Bacteria, Virus and Spore Recovery from Treated OWASA Water (10L) by Conventional & Modified Fresenius F200A HFUFs

Organism	Conventional			Modified		
	Flowrate (L/min)	Trials (N)	Average Recovery (%)	Flowrate (L/min)	Trials (N)	Average Recovery (%)
E. coli K011	0.17±0.02	6	112±36	0.46±0.04	13	60±21
Coliphage MS-2		6	109±18		13	85±12
Bacillus atrophaeus		5	71±19		13	57±13

No Significant Difference by Mann Whitney Test for *E. coli*, coliphage MS-2 and *Bacillus atrophaeus*; p values of 0.0874, 0.5789, and 0.5663, respectively.

Flow rate was significantly greater for HFUFs with modified endcaps (Mann Whitney Test; p value <.0001)

Microbe Recovery from Water using Once-through Gravity HFUF

- Gravity flow HFUF, ca. 30 cm long, 2 cm diameter, 20 nm pore size filter
- 10 L volumes of dechlorinated drinking water
- Spike with high concentrations of *E. coli* K011 (bacterium), coliphage PRD-1 (indicator virus), and spores of *Bacillus atrophius* (protozoan surrogate)
- Filter by gravity flow (1 meter head) or with a peristaltic pump
- Recover test microbes from filter by backflushing with buffered elution solution
  - Used two successive flushes of ca. 250 mL each

Microbial Recoveries from 10L Volumes of Water by Once-through HFUF

- Average recoveries by gravity flow:
  - *E. coli* K011 = 90%,
  - PRD-1 ~100%
  - *Bacillus atrophius* spores = 74%
- Recoveries using a peristaltic pump:
  - *E. coli* K011 = 48%,
  - PRD-1 = ~100%
  - *Bacillus atrophius* spores = 52%

PEG (Polyethylene Glycol) Precipitation of Viruses in HFUF Retentates

- Widely used for virus concentration
  - Protein precipitation
- Minimal virus inactivation; no extreme pH changes
- Secondary virus concentration methods need to be compatible with detection by both molecular and infectivity methods
- PEG precipitation has not been adequately evaluated or optimized for Adenoviruses, Noroviruses and Echoviruses
  - Evaluate effects of PEG and NaCl concentrations on method recovery of these viruses from HFUF retentates and adsorbent filter eluates

Effects of PEG & NaCl Concentrations on Adenovirus Recovery from Treated and Source Water Retentates

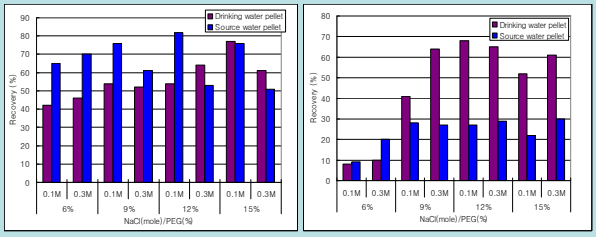
PEG	NaCl	Ad 41			Ad 2		
		N	Pellet	supernatant	N	pellet	supernatant
6%	0.1M	2	81±16	16±1	2	24±30	33±13
	0.3M	2	176±87	17±4	2	63±86	7±1
9%	0.1M	2	107±83	12±5	2	61±83	4±1
	0.3M	2	108±89	12±5	2	59±78	4±1
12%	0.1M	2	92±28	8±8	2	51±68	3±1
	0.3M	2	139±72	19±23	2	29±37	3±1
15%	0.1M	2	51±1	6±5	2	30±40	5±1
	0.3M	2	56±6	6±5	2	31±40	5±1

Treated Water

PEG	NaCl	Ad 41			Ad 2		
		N	Pellet	supernatant	N	pellet	supernatant
6%	0.1M	2	14±7	7±8	2	43±1	15±21
	0.3M	2	126±25	22±21	2	104±28	0±0
9%	0.1M	2	4±1	2±2	2	65±40	7±10
	0.3M	2	50±21	1±1	2	57±10	0±0
12%	0.1M	2	7±8	1±1	2	62±64	0±0
	0.3M	2	55±32	0±0	2	28±11	0±0
15%	0.1M	2	2±1	0±0	2	59±64	0±0
	0.3M	2	28±21	0±0	2	19±15	0±0

Source Water

Echovirus 12 and MS2 Recovery (%) by Different PEG Precipitation Conditions



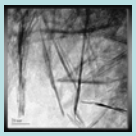
Echovirus 12 (Duplicate trials each) MS2

Conclusions for PEG Precipitation from HFUF Retentates

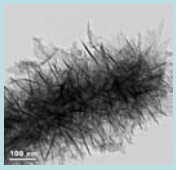
- Effective for secondary virus concentration
- Higher virus concentrations in PEG pellets than in supernatants after centrifugation
- PEG-concentrated PEG samples were compatible with virus detection by both molecular and cell culture infectivity methods
- Overall, 9% or 12% PEG with either 0.1 or 0.3 M NaCl are effective conditions;
  - 0.3 M NaCl better than 0.1 M for Ad 41 in source water
- Virus recoveries by PEG precipitation were more variable from source water retentates compared to those from drinking water retentates

Argonide Nanoceram Electropositive Filter

- Nanoceram filter (Argonide Corporation, Sanford, FL)
- Recently developed electropositive filter
- Reportedly unaffected by pH and salinity of water
- Made from nano alumina (AlOOH) fibers, 2 nm diam. & 0.3 μm long; grafted to microglass fibers; made like paper; 5" pleated cartridge
- External surface area about 500 m^2 per gram of material to provides a large area for adsorption of electronegative particles



Nanoceram alumina fibers



Filter and Water Sources

- Nanoceram filter and filter housing
- Challenge with 40 L of viruses-seeded water
- 10^{10} PCR units of adenovirus
- 10^{10} RT-PCR units of coliphage Qβ
- 10^{6} RT-PCR units of Norovirus GII.4
- 10^{6} murine norovirus
- Source and finished water from drinking water treatment plant in Carrboro, NC.
- Finished water dechlorinated with sodium thiosulfate
- Filter at 25 L/min

Beef Extract Elution of Adsorbed Viruses

- Elution medium: 3% BE (Powder, Becton-Dickinson and Company, Sparks, MD), 0.1 M glycine and with the pH adjusted to 9.5.
- A 500 mL volume was recirculated through the cartridge filter using a peristaltic pump at a flow rate of 1.25-2.75 L per minute
- Flow direction changed every 5 min
- pH monitored
- Final eluent adjusted to pH 7.3

Viral Nucleic Acid Extraction

- Chemical extraction from 100 μL sample volumes
- Guanidinium thiocyanate (GuSCN) extraction via Boom et al. (1990).
- Extract applied to a HiBind RNA minicolumn (OMEGA Bio-Tek, Doraville, GA) and centrifuged at 16,000 x g for 1 minute.
- Columns with nucleic acid washed 2X with 75% ethanol
- Nucleic acids eluted from column with nuclease free water
- Stored at -80° C until analysis.

Virus Quantification by Real-Time PCR

- Previously described real-time PCR quantification:
  - adenovirus 41 (Jothikumar et al, 2005)
  - norovirus (Jothikumar et al, 2005)
  - murine norovirus (Bae and Schwab. 2008)
  - coliphage Qβ (Kirs and Smith. 2001)
- Quantitech probe PCR & RT-PCR kits (Qiagen, Valencia, CA)
  - Reaction volume = 25 µL; 2 µL of extracted viral nucleic acid.
- Smart Cycler thermocycler (v. 2.0c, Cepheid, Sunnyvale, CA).
- Calibration curve used to calculate virus particles (VP) based on cycle threshold value (Ct) created from ten-fold serial dilutions of viral stocks
  - Adenovirus: VP/2µL = 10(-0.2814 * Ct value + 12.256) (R^2 = 0.9986)
  - Norovirus: VP/2µL =10(-0.2726 x Ct value + 10.362) (R^2 = 0.9988)
  - Murine norovirus: VP/2µL = 10(-0.239 x Ct value + 10.41) (R^2 = 0.990)
  - Qβ: VP/2µL = 10 (-0.306 x Ct value + 13.266) (R^2 = 0.996)
- Total VP calculation: Total VP =VP/2µL x 250 x vol. of spike, filtrate or BE solution (in mL)
- Adsorption efficiency: [1-(total VP in the filtrate/total VP in the spike)]*100
- Elution recovery: (total VP in eluent/total VP in spike)*100

Virus Recovery from Source Water using Nanoceram Filter

Virus	% Ads.	% Recovery	# Trials
Adenovirus 41	81% (± 2.4%)	2.4% (± 0.48%)	4
Q_β Coliphage	53% (± 29%)	10% (± 2.8%)	4
Murine Norovirus	74% (± 18%)	9.8% (± 3.3%)	3

Virus Recovery from Finished Water using Nanoceram Filter

Virus	% Ads.	% Rec.	# Trials
Ad 41	97% (± 2.1%)	1.4% (± 0.59%)	8
Q_β coliphage	95% (± 0.86%)	36% (± 20%)	8
Norovirus	ND	26.8%	2

Effect of Tween 80 on BE Elution of Norovirus GII.4 Adsorbed to Nanoceram Filters

Elution of noro GII.4 using 3% beef extracts and a peristaltic pump

Eluent	Estimated norovirus input	Elution replicates % recovered				Average % norovirus recovered
3% BE	$3.5x10^6$	86%	88%	133%	139%	111% (± 29%)
3% BE, 0.1% Tween 80	$3.5x10^6$	95%	140%	99%	141%	119% (± 26%)
3% BE, 0.01% Tween 80	$3.5x10^6$	99%	53%	103%	98%	88% (± 24%)

Ad41 and Norovirus GII.4 Recovery by PEG Precipitation from Nanoceram Filter Eluates

Mean % recovery of Ad 41 and noro GII.4 from eluates by PEG precipitation (n=3)

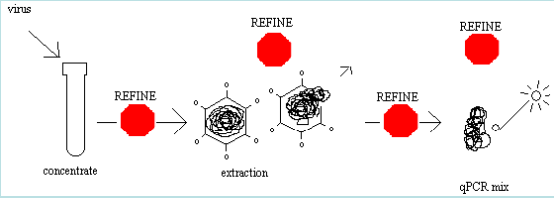
	6% PEG 0.1 M NaCl	6% PEG 0.3 M NaCl	9% PEG 0.3 M NaCl	9% PEG 0.3 M NaCl
Adenovirus 41	1.7% (± 0.14%)	2.9% (± 1.0%)	36% (± 2.3%)	39% (± 6.6%)
Norovirus GII.4	5.6% (± 1.1%)	5.4% (± 0.46%)	52% (± 7.8%)	59% (± 4.8%)

- Higher mean % recoveries of both viruses using 9% instead of 6% PEG (unpaired *t*-test, $p < 0.05$)
- Mean % recoveries not significantly different between 0.1 M and 0.3 M NaCl for Ad41 (unpaired *t*-test, $p = 0.078$) or Noro GII.4 (unpaired *t*-test, $p = 0.122$)

(RT-PCR) Inhibitor Removal and Control in PEG Concentrates

- Substances in virus concentrates inhibit PCR
  - Humic and fulvic acids
  - Other organic compounds
    - proteins, polysaccharides, polyphenols, glycoproteins, etc.
  - Metals
  - etc.
- Quantitative real-time PCR is especially sensitive to such inhibition
- Various methods are available to separate viruses and viral nucleic acids from inhibitors

Sample Processing Steps at which to Remove/Separate/Block Inhibitors



- **Prior to nucleic acid extraction**
- **During nucleic acid extraction**
- **After nucleic acid extraction**
- **During nucleic acid (RT-)PCR amplification**

PEG Samples and Viruses

- PEG concentrates from 40-L water samples processed by Nanoceram filter adsorption-elution (beef extract)
- 3 mL of composite concentrate, added 10 µL of adenovirus, norovirus, and MS-2 stocks
  - virus levels: $9.2x10^8$, $2.8x10^4$ and $5.2x10^8$ PCR units
- Viruses also spiked into 3 mL of PCR grade deionized (DI) water. (Dracor) as a inhibitor-free control sample
- Both PEC concentrate and DI control processed
- qPCR CT values of PEG and DI control samples were compared to calculate ΔCt values
  - $\Delta Ct = CT_{Sample} - CT_{DIcontrol}$
  - Smaller ΔCt: less inhibition
  - Larger ΔCt: more inhibition

Treatments before NA Extraction with GuSCN

- Sephadex G-200 column chromatography
  - High salt TE buffer to prepare columns
  - Biospin polypropylene columns
    - Bio-Rad Cat. #732-6204, 3 cm, 0.8 ml capacity
  - 1 mL polypropylene syringe column (BD) with sterile glass wool (Supelco)
- Chelex 100 + Sephadex G-200 columns
  - Chelex in bottom half; G-200 in top half

Modifications during nucleic acid extraction

- GuSCN extraction of different sample volumes
  - 400, 300, 200, 100, and 50 µl samples
- Chloroform extraction of 300 µL & 100 µL sample volumes
  - 1:1 volume ratio
- Polyvinylpyrrolidone (PVP)-GuSCN extraction
  - 1% final concentration of PVP in sample-GuSCN mix

Post-extraction Modifications

- Isopropanol precipitation of NA Extract
  - Sample NA extract supplemented with Na acetate and isopropanol; centrifuged; NA ppt. washed with 70% EtOH; centrifuged; NA ppt. dried, then resuspended in water

qPCR Methods

Adenovirus: JTVXF primer, JTVXR primer, JTVXP probe
- Jothikumar and Cromeans (2005).

- Norovirus: JJGII primer, COG2R primer, Ring2-TP probe
  - Jothikumar and Lowther (2005)
- MS-2: ms2ks2 primer, ms2ks1 primer, ms2ks3 probe
  - Bae and Schwab (2008)
- Smart Cycler (Cepheid)

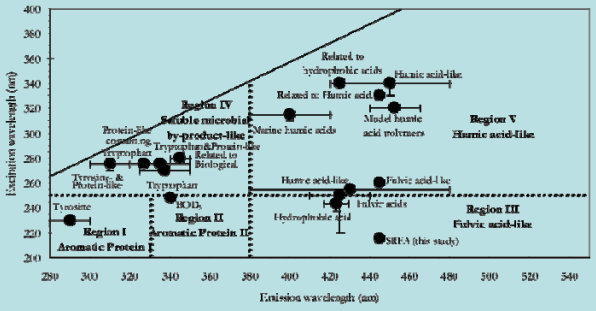
Modifications to qPCR Mix

- Add PVP
- Add PVP and glycerol
- Add Bovine Serum Albumin (BSA)

Fluorescence Spectrophotometry:

- Fluorescent excitation emissions matrix (EEM) to quantify dissolved organic matter
- Detects and differentiates humic acids, fulvic acids, tryptophan and other potential organic inhibitors
- Sample run included quinine hemisulfate stock solutions for calibration and reagent grade water for comparison and background subtraction

EEM Peak Regions, Based on Excitation (Y-axis) and Emission Wavelengths (X-axis)



Treatments for qPCR Inhibitors

- No treatment before, during, or after extraction of viruses concentrated from water samples improved viral detection by qPCR with the same effectiveness for adenovirus, norovirus, and MS-2 in PEG concentrates of surface water samples
- Different methods or treatments may be needed for each type of water sample and virus.
- Specific treatments were more effective in lowering delta ct values for qPCR detection of viruses in many of the samples.

qPCR detection of three viruses in different water sample volumes subjected to chloroform extraction relative to detection in reagent water

	Adenovirus			Norovirus			MS-2		
Modification	ΔCt value	ΔCt st dev	p	ΔCt value	ΔCt st dev	p	ΔCt value	ΔCt st dev	p
$CHCl_3$ 300	7.23	0.539	<0.01	8.62	0.992	<0.01	5.50	0.334	<0.01
$CHCl_3$ 100	2.96	0.309	<0.01	3.34	0.479	<0.01	5.15	0.715	<0.01

Comparison of different surface water sample volumes subjected GuSCN extraction for differences in qPCR detection of adenovirus and norovirus relative to detection in reagent water

	Quasi-Point Source-Impacted Water						Non-point Source-Impacted Water					
	Adenovirus			Norovirus			Adenovirus			Norovirus		
Sample Volume	ΔCt value	ΔCt st dev	p	ΔCt value	ΔCt st dev	p	ΔCt value	ΔCt st dev	p	ΔCt value	ΔCt st dev	p
400 µL	5.84	0.50	<0.01	7.57	0.288	<0.01	5.71	0.57	<0.01	7.57	0.28	<0.01
300 µL	4.80	0.68	<0.01	7.91	2.06	<0.01	7.79	2.79	0.0113	3.03	0.52	<0.01
200 µL	4.96	0.82	<0.01	6.52	0.759	<0.01	5.10	0.34	<0.01	2.68	0.86	<0.01
100 µL	5.33	0.85	<0.01	3.59	0.700	0.06408	5.00	9.35*	0.1280	1.39	2.60	0.5500
50 µL	4.37	1.78	0.0116	2.47	0.770	<0.01	4.18	0.62	<0.01	0.10	0.21	0.6465

Most Effective Sample Treatments

- Sephadex G-200 followed by chloroform extraction
  - Best for adenovirus in NPS water sample
  - Best for MS-2 in quasi-PS water sample
- Chloroform extraction alone
  - Good for norovirus in NPS water sample.
  - Best for MS-2 in NPS water sample
- GuSCN extraction of smaller sample volume
  - Best for norovirus in both samples
- Sephadex G-200 and Chelex 100 treatment
  - Best for adenovirus in quasi-PS water sample

Overall Summary

- Primary virus concentration by improved recirculating UFUF is effective and rapid
- Primary virus concentration by once-though HFUF shows promise
- Primary virus concentration by Nanoceram filters is effective and very rapid but less effective than desired for adenoviruses
- PEG precipitation is effective for 2nd step virus concentration
- PEG sample treatments prior to nucleic acid extraction reduce sample inhibition and improve virus detection by qPCR

Thank-you!

Questions? Comments? Suggestions?

Collaborators:

Erik Andersen
Lisa Casanova
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Lauren Thie
Jan Vinjé
Jianyong Wu
Ming Jing Wu

Additional $ Support:

AWWARF
NOAA – CICEET; NERRS
NWRI
SCCWRP
UNC Sea Grant

Quantitative Assessment of Pathogens in Drinking Water

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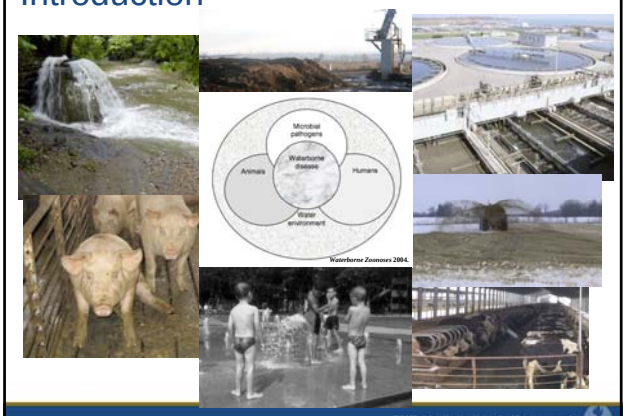
Introduction



Microorganisms in Source and Finished Water

Microbial contaminants can be divided into 3 categories:

1. Parasites
2. Viruses
3. Bacteria

KEY concepts to keep in mind

1. Size of the microorganism
   Parasites > Bacteria >> Viruses
2. Resistance to environmental degradation and chemical inactivation
   Parasites > Viruses >> Bacteria

Waterborne Pathogens and Gastroenteritis

Etiologies of Waterborne Outbreaks, 1991-2002

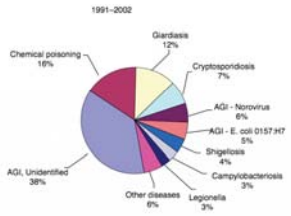


Figure taken from Craun 2006; AGI=acute gastrointestinal illness

- On average, between 1991 and 2002, 17 waterborne disease outbreaks (WBDOs) were reported annually.
- 38% of outbreaks had an unidentified etiology
- WBDOs were primarily associated with inadequately treated water systems and contamination issues related to aging distribution systems
- In some instances, the water systems were in compliance with **current water quality standards**

Waterborne Pathogens and Gastroenteritis

- Multiple Factors Influence Reporting of AGI
  - Public awareness of waterborne illnesses
  - Local requirements for reporting cases of particular diseases
  - The surveillance and investigative activities of state and local public health and environmental agencies
  - Availability of and extent of laboratory facilities
- Current waterborne disease surveillance system is passive
  - Waterborne disease outbreaks are likely to be under reported
  - Endemic waterborne disease risk in the United States is not well understood

Craun *et al.* 2006

Why is all of this of interest?

One of the major limiting factors in assessing microbial loads in source and treated drinking water has been the **lack of an effective microbial collection method capable of efficiently and simultaneously recovering low levels** of bacteria, viruses and protozoa, which then can be identified and quantified rapidly with or without cultivation.

Research Objective

Develop rapid, sensitive recovery and detection methods for the quantitative assessment of pathogenic microorganisms present in drinking water.

Microbial Recovery

Develop and optimize sensitive concentration and isolation methods utilizing filtration technology capable of simultaneously recovering low levels of protozoa, viruses, and bacteria from large volumes of water.

- Demonstrate ability of tangential flow filtration (TFF) to efficiently recover/concentrate intact microorganisms from water
- Determine lower limit of detection for each class of microorganism

Steps for Processing 1 to 1,000 L Water Samples

Microbial Surrogates Utilized in Method Evaluation

- Vegetative Bacteria
  - *Escherichia coli* CN-13
  - *Enterococcus faecalis*
- Spore-forming bacteria
  - *Clostridium perfringens*
- Bacteriophage
  - MS2
  - PRD1
- Viruses
  - Murine norovirus (MNV-1)

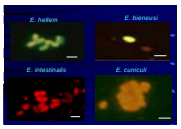
Pathogen Detection

Develop rapid, quantitative molecular detection techniques for the identification of target pathogens including direct comparison with existing traditional culture methods.

- Optimized FISH methods for the identification of protozoa.
- Developed mass spectrometry (MS) methods for the identification of select microorganisms.
- Refined qPCR and qRT-PCR assays for the detection of select microorganisms.
- Developed loop-mediated isothermal amplification (LAMP and RT-LAMP) for the detection of select microorganisms.
- Employed the use of internal standard controls for the detection of PCR inhibition caused by molecular inhibitors present in water samples.

Fluorescent ***In Situ*** **Hybridization** (FISH)

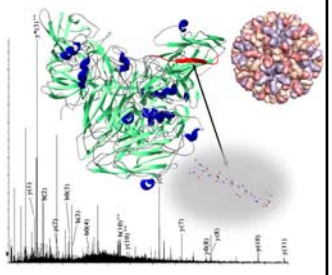
- Employs a fluorescently labeled oligonucleotide probe targeting species-specific sequences of 16S rRNA
- rRNA
  - Exists in multiple copies
    - Present in high copy numbers in ***viable*** cells
  - Single-stranded regions allow easy access for the probe and natural signal amplification



- Hybridization
  - Probes recognized by fluorescent antibodies
- Observed under epifluorescence microscope

Detection using Mass Spectrometry (MS)

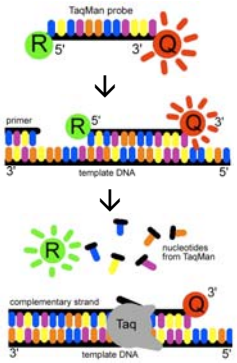
- Detect capsid protein
  - Multi-copy
  - Uniquely identifiable
- Purify protein
- Digest with protease (trypsin)
  - Spike peptide standards
- Chromatographically separate peptides and then determine amino acid sequence
- Search masses against genome databases (e.g. NCBI)
- Assess confidence-based score
- Quantification



MS Key Findings – Norovirus

- **The NV capsid protein is detectable in the clinical range using MALDI-TOF MS**
- **Clinical sample complexity requires a more nuanced approach (ESI-MS/MS)**
- **Using additional sample processing, MS/MS methods can improve sensitivity by 2-3 orders of magnitude**
- **AQUA peptides allow for the quantification of peptides from capsid protein of norovirus**

Molecular Methods: Real Time PCR



- DNA is amplified by a cycling of steps:
  - Denaturation
  - Primer annealing
  - Primer extension
- TaqMan™ probe technology allows for real time quantification of target RNA/DNA
  - Fluorescent probe is cleaved during extension
  - Target is quantified in the form of a cycle threshold (C_t) value

http://www.bio.davidson.edu/Courses/Molbio/MolStudents/spring2003/Pierce/realtimepcr.htm

Molecular Methods: LAMP

- Loop-mediated isothermal amplification (LAMP) is a novel detection method which relies on auto-cycling strand displacement DNA synthesis.
  - RT may be used in conjunction for detection of RNA viruses
- Increased sensitivity and specificity compared with conventional PCR
  - Multiple primers must recognize several distinct regions on the target RNA/DNA
- Products can be analyzed in real time by measuring the increase in turbidity during DNA amplification.
  - Allows for real time quantification

Field Application

Apply tangential flow ultrafiltration and quantitative molecular detection to large-volume, water samples for the analysis of microorganisms.

- Spike environmental water samples with microbial surrogates to evaluate the efficiency of recovery and detection methods.
- Apply complete concentration, recovery, and detection process to a variety of water samples including ground water, surface water, and finished drinking water.
- Compare newly developed technologies for the recovery and detection of microorganisms in water to existing US EPA methods.
- Identify viruses that are endemic and stable in the environment and investigate their use as traceable markers of fecal contamination

Collection of Environmental Samples

- Water sampling in Lower Yakima Valley, WA
  - Sampled surface water and ground water impacted by surrounding dairy industry
  - Application of optimized TFU method for concentration of 100L water samples

Water Sampling in Yakima Valley, WA

- Processing
  - Applied optimized TFU in the field
  - Seeded each sample with known concentration of MNV-1 to evaluate recovery efficiency
- Parameters
  - Utilized a Multiparameter Water Quality Sonde
    - Temperature, turbidity, pH, conductivity, dissolved oxygen, nitrate, and nitrite
- Analysis
  - IDEXX Most Probable Number (MPN) method
    - Total coliforms, *E. coli*, and *Enterococcus*
  - FISH/IFA, qPCR/RT-PCR
    - Ongoing for select protozoa and viruses

Surface Water (n=11)
Groundwater (n=10)

Public Health Implications

- Developing a universal method for the recovery of microorganisms will enable water utilities and regulatory agencies to better address problems within source waters and public water systems.
- The utilization of molecular detection techniques will provide increased confidence in the sensitivity, specificity, and inhibition detection/control critical for estimating levels of risk.
- A more comprehensive understanding of the microbial contamination of water sources will allow for exposure risk assessments to be generated for individual microorganisms
- Future applications of this method:
  - Further the development of the usefulness of host-specific viruses in microbial source tracking efforts
    - Currently limited by lacking concentration and detection methods
  - Assist in the formulation of effective control measures for the reduction of water-related transmission of pathogenic microorganisms

Acknowledgements

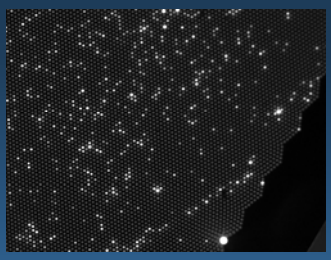
Collaborators

Drs Rolf Halden, Thaddeus Graczyk

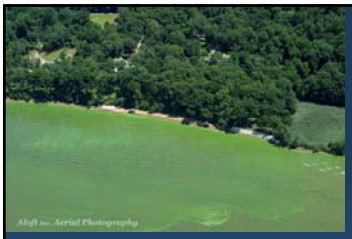
Students – Kristen Gibson, David Colquhoun

Funding – EPA STAR R833002

Development and application of a fiber optic array system for detection and enumeration of potentially toxic cyanobacteria



Donald M. Anderson
Woods Hole Oceanographic Institution, Woods Hole, MA



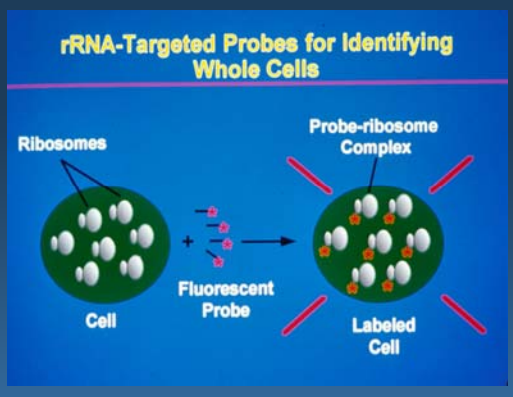
The problems:

- **Many cyanobacteria produce potent toxins that threaten human health**
- **CyanoHABs can take multiple forms, ranging from dense surface scums to dilute suspensions that can still cause harm.**
- **Many different species and strains co-occur, and strains of the same species can be toxic or non-toxic, or can vary dramatically in the amount of toxin produced under different conditions.**
- **Distinguishing characteristics can be difficult to discern under the light microscope, yet such fine levels of discrimination are not feasible in monitoring programs that generate large numbers of samples.**

The overall project goal is to adapt and validate a rapid and accurate optical fiber-based technology for cyanoHAB cell detection and enumeration in both laboratory and field settings.

Specific objectives are to:

1) **Design rRNA signal and capture probes for the three most important toxic cyanobacteria (*Microcystis aeruginosa, Cylindrospermopsis raciborskii, and Anabaena flos-aquae*);**
2) **Design and test a second probe pair for each species, to incorporate redundancy into the array;**
3) **Test these probes in the fiber-optic array format and determine detection limits, specificity, and dynamic range;**
4) **Refine hybridization conditions to reduce processing time;**
5) **Develop procedures to analyze multiple cyanoHAB species simultaneously using a single fiber bundle in a multiplexed format**



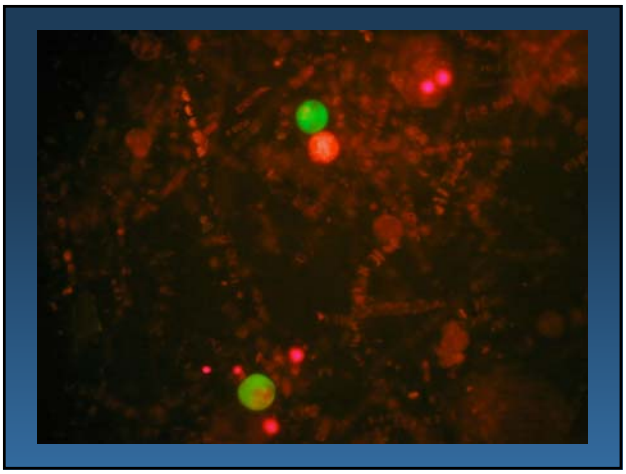
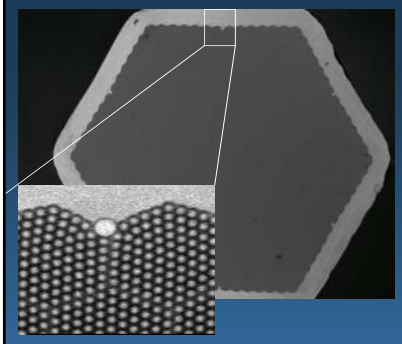
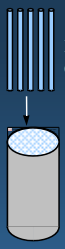


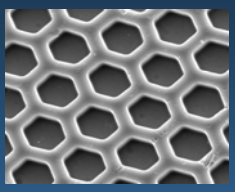
Image of Fiber Bundle



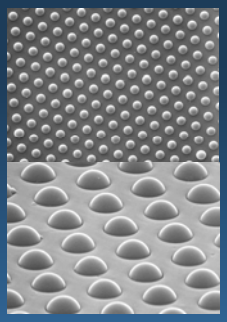


Epstein, J. R. and Walt, D. R., Chem. Soc. Rev., 2003, 32, 203-214

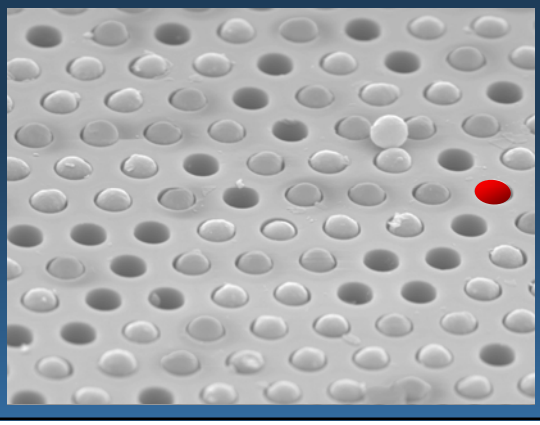
Microspheres in Etched Wells



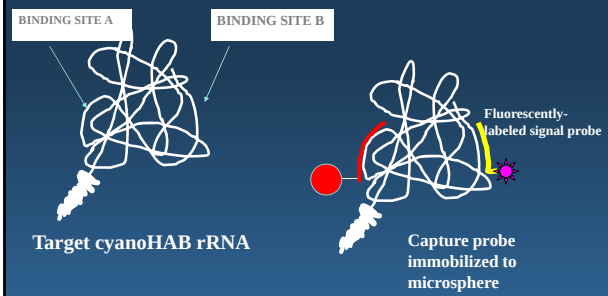
Wet etching with HCl



Pantano, P.; Walt, D.R. *Chem. Mater.* **1996**, 8, 2832-2835

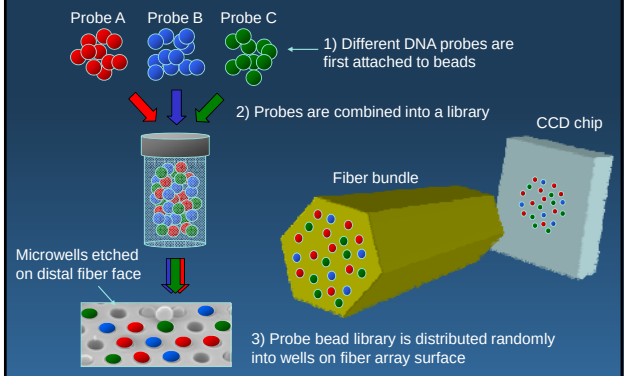


HAB rRNA Sandwich Assay

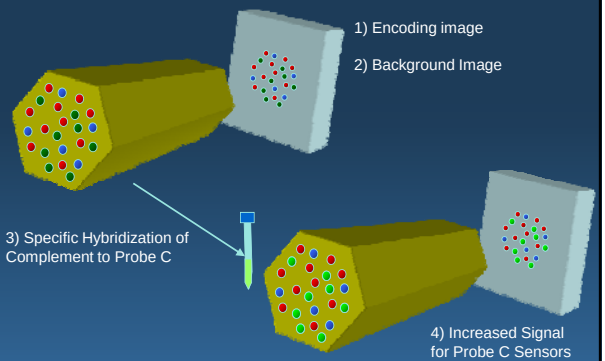


Nicholls and Malcolm, Nucleic acid analysis by sandwich hybridization. *J. Clin. Lab. Anal.* 1989; 3(2), 122-135.

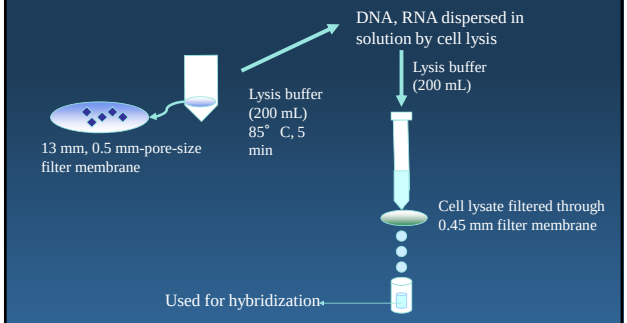
Optical Fiber-based Microsphere Array



DNA Hybridization

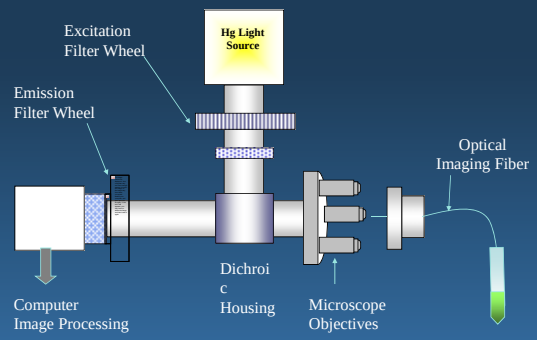


rRNA sample preparation

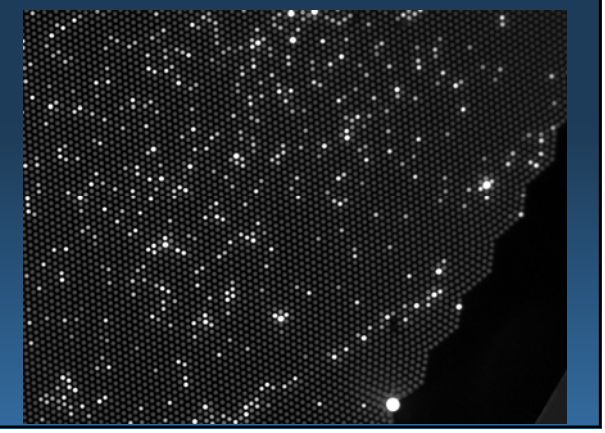


==> No RNA purification needed

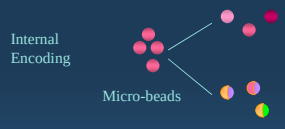
Fiber optics instrumentation



Imaging System



Encoding strategies for multi-species detection: an optical bar code

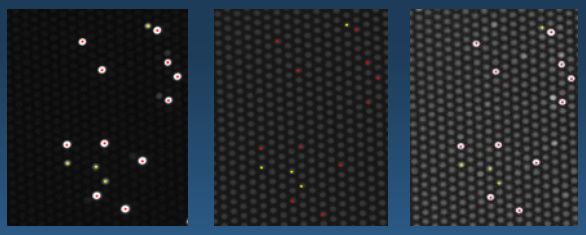


Number of dyes: M
Concentration levels of each dye: n
Number of "optical bar code options": M^n

e.g. 4 colors, 5 concentration levels of each, could detect 4^5=1024 species.

==> Theoretically, one sensor array can be designed to detect and enumerate all HAB species in a given region

Images from DNA Sensor Array

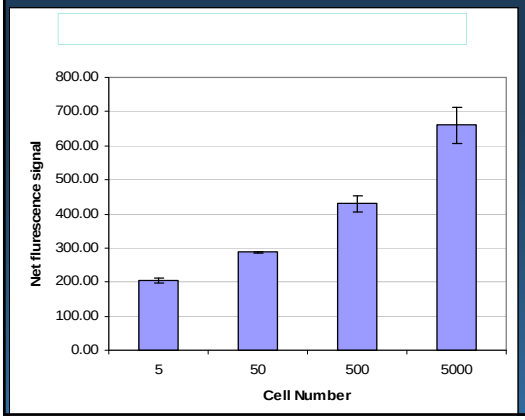


Encoding image
- BA2
- YP2

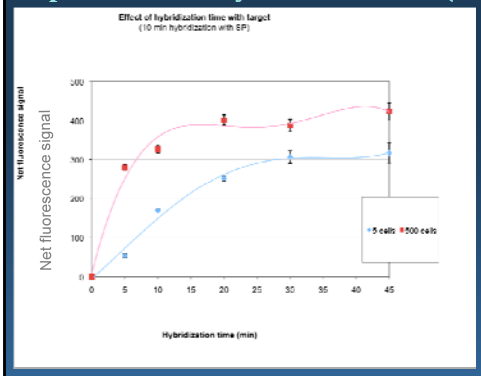
Background before hybridization

Hybridization with BA2 target,10 min,

Detection limit for *Alexandrium fundyense*

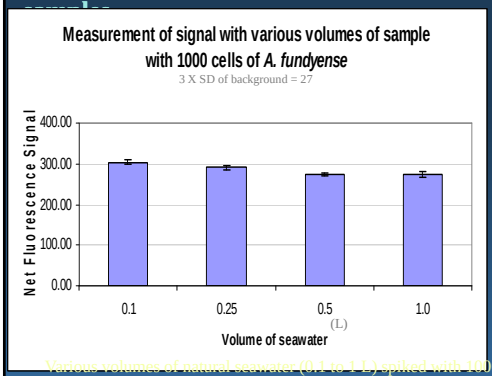


Optimization of hybridization time (no stirring)



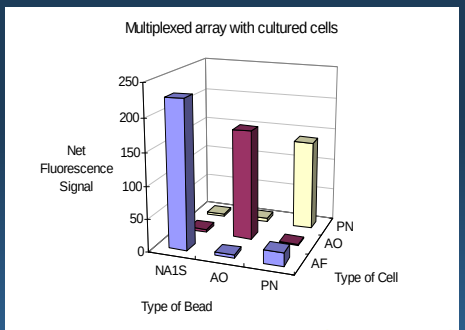
→Lower number of cells need longer hybridization time to reach saturation

Detection of *A. fundyense* cells in natural seawater



Various volumes of natural seawater (0.1 to 1 L) spiked with 1000 cells of *A. fundyense*.

Multiplexed array with 3 sensors



- Multiplexed array was tested with cultured cells of 3 organisms (*A. fundyense*, *A. ostenfeldii*, and *Pseudo-nitzschia australis*)
- Each sensor showed positive signal only with its target organism - no significant cross reactivity

Application to cyanoHABs

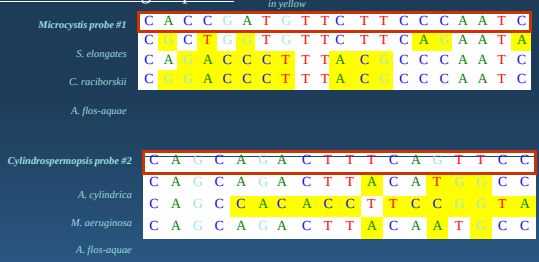
Methods

- **Signal and capture probe design**
  - **16S rRNA gene sequences compiled from GenBank for target cyanoHAB taxa: *Cylindrospermopsis raciborskii, Microcystis aeruginosa,* and *Anabaena flos-aquae***
  - **Probe identification performed using sequence alignments of target/non-target species**
  - **Included published probes for *Microcystis, Anabaena/Aphanizomenon,* and *"Nostoc group" (Nostoc/ Anabaena/Aphanizomenon)***
- **Probes tested against target and non-target species using fluorescent in situ hybridization (FISH) to determine efficacy and assess cross-reactivity; probes that exhibit cross-reactivity require re-design**
- **Probes successfully tested for cross-reactivity are then transitioned to fiber-optic microarray format and tested against synthetic target and cell lysates from target species**

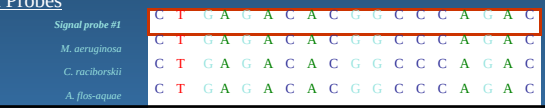
Probe design

Capture Probes for Target Species

Base pair mismatches for capture probe development are highlighted in yellow



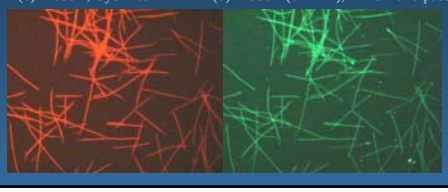
Signal Probes



Cylindrospermopsis raciborskii LB 2897

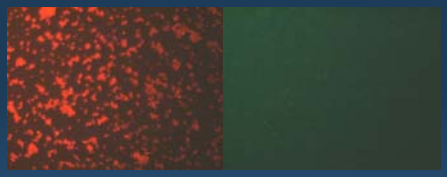
(a) No probe, Cy3 filter (b) No probe, FITC Band pass filter

(c) Probe 2, Cy3 filter (d) Probe 2 (6-FAM), FITC Band pass filter

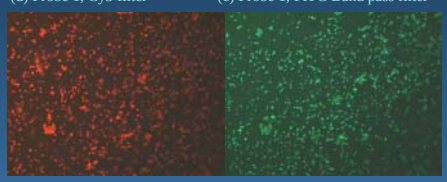


M. aeruginosa LE-3

(a) No probe, Cy3 filter (b) No probe, FITC Band pass filter

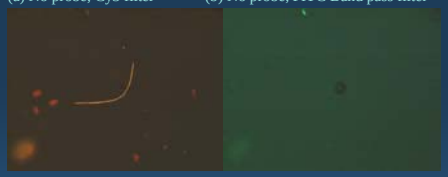


(d) Probe 1, Cy3 filter (e) Probe 1, FITC Band pass filter

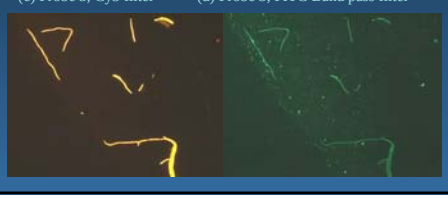


Anabaena flos-aquae LB 2557

(a) No probe, Cy3 filter (b) No probe, FITC Band pass filter



(c) Probe 3, Cy3 filter (d) Probe 3, FITC Band pass filter



Cross-reactivity testing (in progress)

Probe Number	CYL 1	CYL 2	ANAB-1*	ANAB 2	ANAB 3	ANAB 4	NOS-ANAB-1*	MICRO-1*	MICRO 2	MICRO 3	MICRO-D4*	MICRO 5
Length (bp)	18	20	30	19	27	22	28	27	20	60.7	26	20
Tm (°C)	50.6	54.9	61.5	58.6	66.3	51.4	57.6	61.5	54%	21	64	59.6
Microcystis sp. 118/2								+	+	+	+	+
Microcystis sp. TN-6								+	+	+	+	+
Microcystis sp. OS-3								-	+	-	+	+
M. aeruginosa LB 2061	-	-	-	-	-				+			
M. aeruginosa LE-3	-	-	-	+	-	-	-	+	+	+	+	+
M. flos-aquae 2673	-	-	-	-	-				+			
C. raciborskii AWT 205	+	+	-	+	+	-	-	-	-	+	-	+
C. raciborskii LB 2897	+	+		-	-			-	-			
C. raciborskii THAI	+	+	-	-	-		+	-	-			
A. flos-aquae LB 2557	-	-	-	+	-				-			
A. flos-aquae LB 2558	-	-	-	+	-		+		-			
A. flos-aquae NH-5	-	-	-	-	-				-			
A. flos-aquae UTEX 2391	-	-	-	+	+	-	-	-	-	-	-	-
Aphan. flos-aquae	-	-	-	-	+	-	-					
A. cylindrica UTEX B 629	-	-	-	-	+	-	-					
A. bergii AZ-73	-	-	-	-	-	-	-					
Anabaenopsis sp. AZ-16	-	+										
Aphanizomenon sp. AZ-10			-	+	+	-	-					
Nostoc muscorum UTEX 1037	-	-	-	-	-	-	-					
Synechocystis PCC 6803								-	-	-	-	-
S. elongatus PCC 7942								-	-	-	-	-
Oscillatoria sp. AZ-40								+	+	+	+	+
Lyngbya sp. 7-10a								-	-	-	-	-
Nodularia sp.	-	-	-	-	-	-	-					
Planktothrix PCC 7811	-	-										
Synechococcus sp. BO8807	-	-								-		-

* Denote published probe sequences

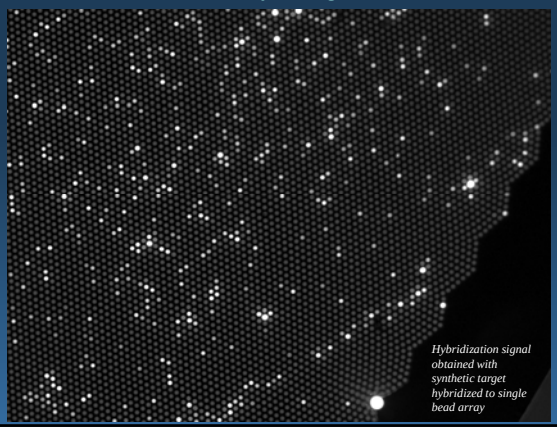
Twelve probes tested for cross-reactivity (in progress)

- ***Microcystis*** **probes (3)**
  - **Tested against 18 cultures (in progress)**
  - **All designed (3) and published (2) probes exhibit cross-reactivity with *Oscillatoria;* redesign in progress**
- ***Cylindrospermopsis*** **probes (2)**
  - **Tested against 18 cultures**
  - **One probe transitioned to fiber optic microarray format**
  - **Second probe exhibited cross-reactivity with *Anabaenopsis*; redesign in progress**
- ***Anabaena*** **probes (5)**
  - **All designed (3) and published (2) probes either exhibited cross-reactivity or failed to detect target species**
  - **Taxonomy of *Anabaena* problematic (not monophyletic); redesign efforts needed to develop probe for *Anabaena/Aphanizomenon* or "Nostoc group"**

Microarray testing

- **Capture probe performance tested using *Cylindrospermopsis* probe #1 (CYL1) coupled to activated microbeads and against a synthetic target**
  - **Single bead array exposed to Cy5-labeled synthetic targets with sequences complementary to the capture probe**
  - **Hybridization was performed at room temperature using 100 µl of synthetic target solution (100 µM) and a hybridization time of 10 minutes**

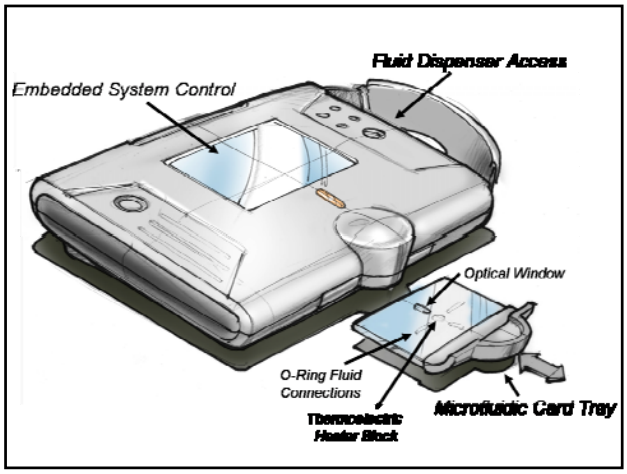
Microarray Testing

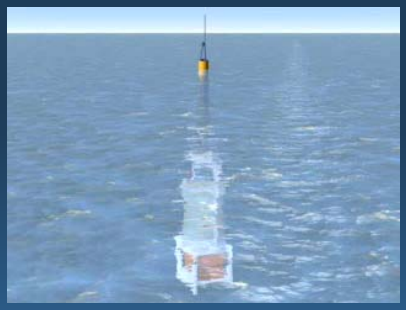


Hybridization signal obtained with synthetic target hybridized to single bead array

Future directions

- **Probe redesign and testing**
- **Transition additional probes to microarray format (single bead arrays) and assess performance using synthetic targets and cell lysates (assess detection limits, specificity, and dynamic range)**
- **Assess performance of multiplexed array using single and multiple species**
  - **single species and mixed cultures**
  - **spiked/unspiked field samples (2009 field sample collections include lakes in OR, MA, MD, CA, FL and Great Lakes)**
- **Explore application of the microarray technique on a portable instrument**
- **Explore remote deployment of the microarray technique on a robotic, in situ instrument**





The Environmental Sample Processor (ESP)

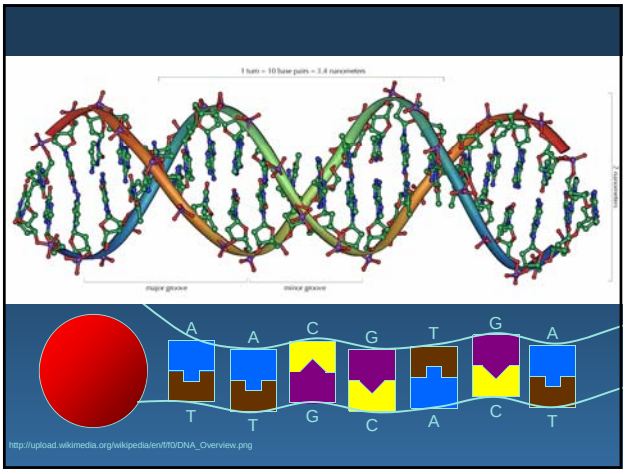
Source: C. Scholin, MBARI

Acknowledgements

Woods Hole Oceanographic Institution
Mindy Richlen
Dave Kulis
Rob Arnold

Tufts University
David Walt
Ryan Hayman
Shonda Gaylord

This research is funded by
U.S. EPA - Science To Achieve Results (STAR) Program
Grant # RD-83382801-0



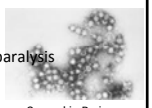
Development of high-throughput and real-time methods for the detection of infectious enteric viruses

JJL Cantera, H-Y Yeh, A Mulchandani, W Chen & MV Yates

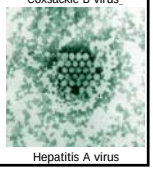
UNIVERSITY OF CALIFORNIA, RIVERSIDE

Human Enteric Viruses

- *Enterics*: adenoviruses, enteroviruses, noroviruses, rotaviruses
  - *Enteroviruses*: coxsackievirus, hepatitis A virus, echovirus & poliovirus
- Can cause serious diseases when ingested
  - *e.g.* gastroenteritis, meningitis, hepatitis, myocarditis, paralysis
- Stable in aquatic environments
- Transmitted by fecal-oral route
- Low infectious dose



Coxsackie B virus



Hepatitis A virus

Human Enteric Viruses

Genus	Popular name	Disease caused
Enterovirus	Poliovirus	Paralysis, meningitis, fever
	Coxsackievirus A, B	Meningitis, fever, respiratory disease, hand-foot-and-[illegible]myocarditis, heart anomalies, rush
	Echovirus	Meningitis, fever, [illegible]ratory disease, rush, gastroenteritis
Hepatovirus	Hepatitis A	Hepatitis
Reovirus	Human re[illegible]rus	[illegible]known
Rotavirus	Human [illegible]virus	G[illegible]enteritis
Mastadenovirus	Human [illegible]novirus	Gastr[illegible]eritis, respirator[illegible]isease, conjunctivitis
Calicivirus	Human c[illegible]ivirus	Gastroen[illegible]
	Norwalk v[illegible]	Gastroenteriti[illegible]ver
	SRSV	Gastroenteritis
	Hepatitis E	Hepatitis
Astrovirus	Human astrovirus	Gastr[illegible]eritis
Parvovirus	Human parvovirus	Gastroenteritis
Coronavirus	Human coronavirus	Gastroenteritis, respiratory disease
Torovirus	Human torovirus	Gastroenteritis

Source: Bosch, A. 1998. International Microbiology 1: 191-196

Detection Methods

Principle of the assay	Example	Infectivity test	Detection limit (particles/ml)	Duration
Visualization of **viral particles**	EM	No	10^5 to 10^6	< 24 hr
Detection of **viral proteins** or **antibodies**	ELISA	No	10^5	< 2 hr
Detection of **viral genome**	Probe hybridization	No	10^4	< 2 hr
	RT-PCR	No	10^1 to 10^3	< 8 hr
Detection of **cytophatic effect**	Plaque assay	Yes	10^0 to 10^1	2 to 14 days

Koopmans & Duizer (2004) Int J Food Microbiol 90: 23-41

Main Aim

To develop methods for high-throughput and real-time detection of infective enteric viruses

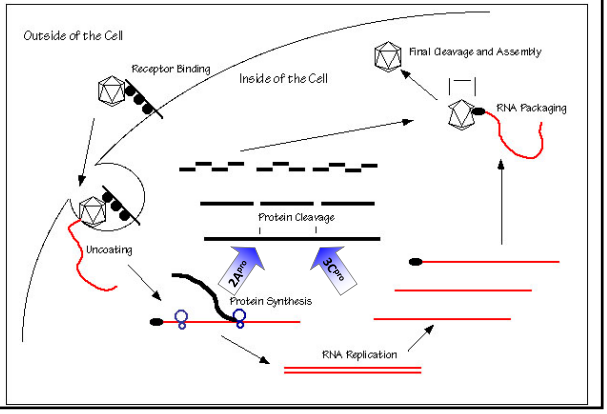
Part 1: Genetically engineered reporter cells

- Viral protease-sensitive fluorescent substrate
- Detects viral protease
- Flow cytometry-based assay for detection of PV in wastewater

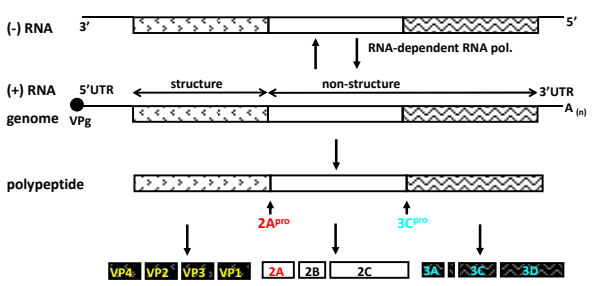
Part 2: Nuclease-resistant molecular beacons (MBs)

- Detects viral genome
- Modified MB for visualizing the dynamics of viral replication in living cells

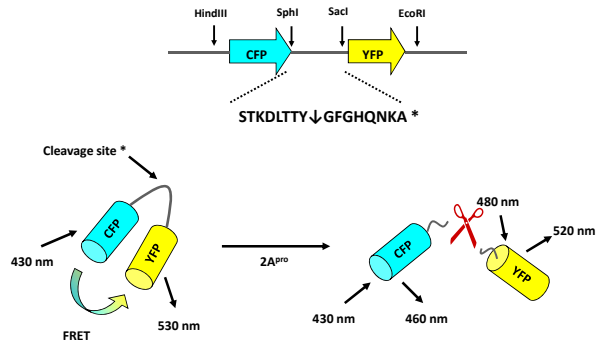
Poliovirus: Life Cycle



Poliovirus: Genome

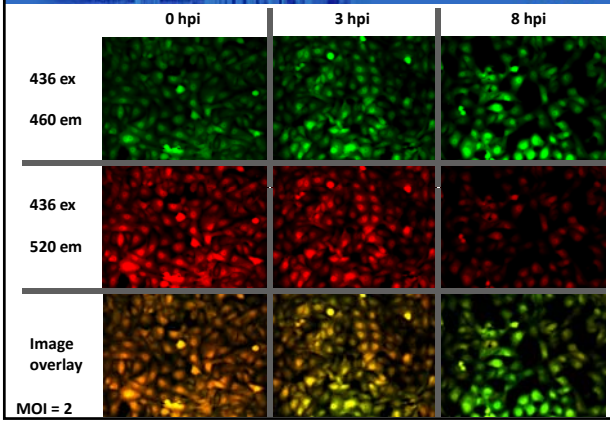


Reporter cells: Construction & Principle



Fluorescence Resonance Energy Transfer

Reporter cells: Microscopic examination



Reporter cells: Fluorescence Activated Cell Sorter

BGM-PV

↓

Infection with PV1

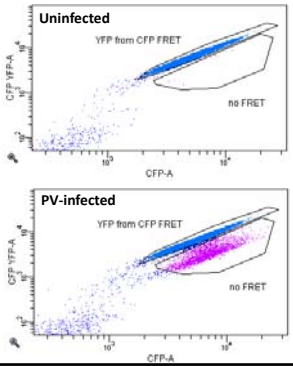
↓

Infected BGM-PV

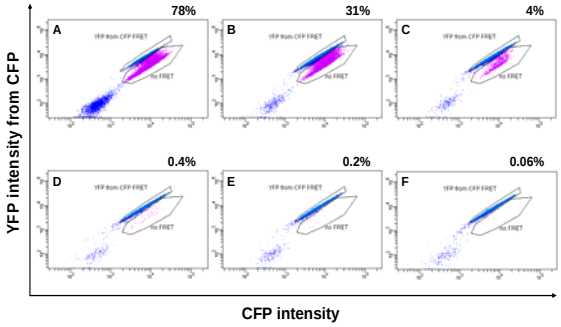
↓

FACS analysis

FACS dot plots



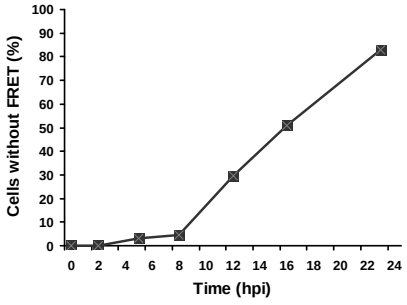
Reporter cells: FACS results



Increase in CFP intensity in PV-infected cells vs uninfected cells

→ FACS can distinguish infected cells from non-infected cells
→ FACS can detect quantitative differences in the number of infected cells in the sample

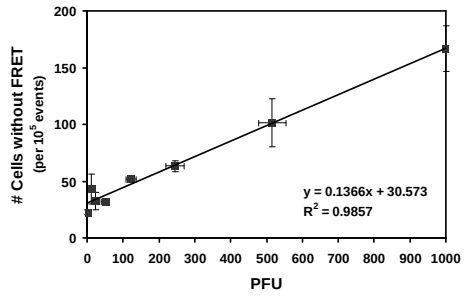
Reporter cells: FACS results



An increasing number of infected cells was detected as PV1 infection progressed

→ FACS assay is suitable for following the kinetics of poliovirus infection
→ FACS can detect infected cells as early as 5 hpi!

Reporter cells: FACS vs. plaque assay

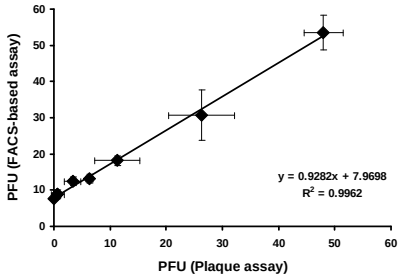


FACS detected cells infected with 50 PFU of PV1 after 8 hpi… positive signals from cells infected with 1 PFU of PV1 were detected after 12 hpi!

→ **FACS is a sensitive and rapid method for PV1 detection**

Reporter cells: FACS vs. plaque assay

When tested in waste water spiked with PV1,



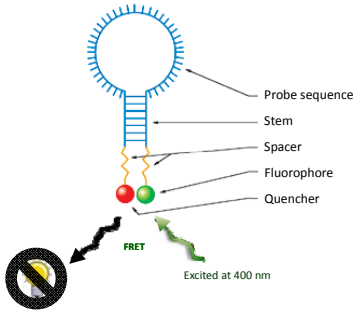
No significant difference between FACS and plaque assay results

→ **FACS is a reliable method for PV1 detection in waste water**

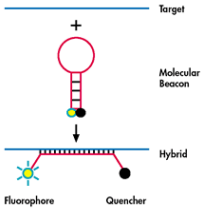
Summary

- Using FACS on fluorescent reporter cells:
  - distinguished infected from uninfected cells
  - detected PV-infected cells as early as 5 hpi (at high infective dose)
  - detected 1 PFU of PV after 12 hpi
  - Good correlation between FACS-based and plaque assays when tested on wastewater spiked with PV1

Molecular Beacons: Structure and principle



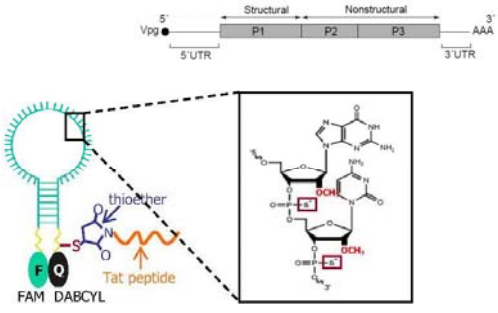
Structure



Principle

Tyagi et al, *Nat. Biotechnol.*, 14, 303-308 (1996)

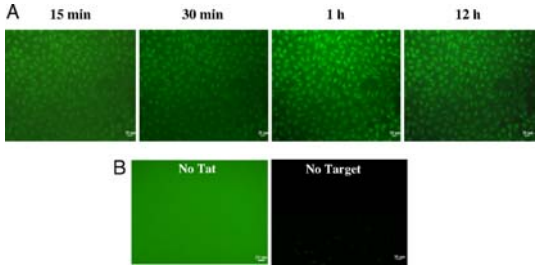
MBs: Design and Modifications



Yeh H. et.al. PNAS 2008;105:17522-17525

PNAS

MBs: Intracellular delivery

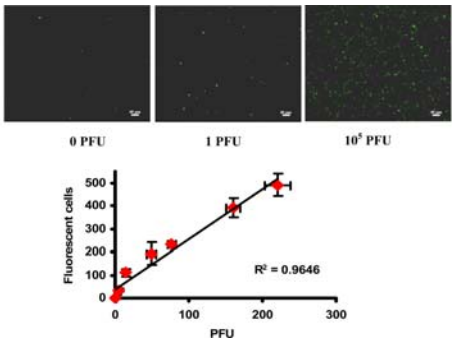


Intracellular delivery of MB CBV6-Tat–target hybrids (A) or MB without Tat modification or without targets (B)

Yeh H. et.al. PNAS 2008;105:17522-17525

PNAS

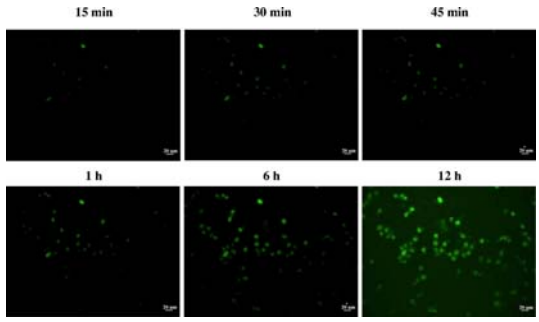
MBs: *In vivo* detection of CVB6



Yeh H. et.al. PNAS 2008;105:17522-17525

PNAS

MBs: Real time detection of viral spreading



Yeh H. et.al. PNAS 2008;105:17522-17525

PNAS

Summary

- Modified molecular beacon
  - Nuclease-resistant MB with TAT peptide was designed
  - Detected as few as 1 PFU during the early stage of viral replication
  - Fluorescence assay was comparable with the plaque assay
  - Used to monitor the dynamics of viral replication during a 12-h infection period

Conclusion & Future Perspectives

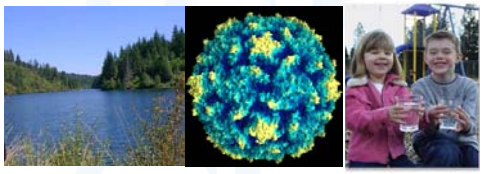
- Developed methods for detecting infective viruses
- Sensitivity could reach 1 PFU at shorter incubation time than conventional plaque assay
- Reliable for viral quantitation
  - Detection of other epidemiologically important viruses
    - e.g., hepatitis virus, adenovirus, norovirus
  - High throughput screening for viral protease inhibitors
  - Development of other FRET-based sensors

Acknowledgment

- Drs. Marylynn Yates and Wilfred Chen
- Members of Yates and Chen Laboratories
- B. Walters (UCR Institute for Integrative Genome Biology)
- U.S. Environmental Protection Agency

- The End -

New Electropositive Filter for Concentrating Enterovirus and Norovirus from Large Volumes of Water



Mohammad R. Karim, Eric R. Rhodes, Nichole Brinkman, Larry Wymer, and G. Shay Fout

Presentation Outline

- Human enteric viruses
- Why should we be concerned about viruses in water
- General method for virus detection
- Research need for virus sample collection
- Evaluation of a new filter for concentrating viruses from water
- Conclusions

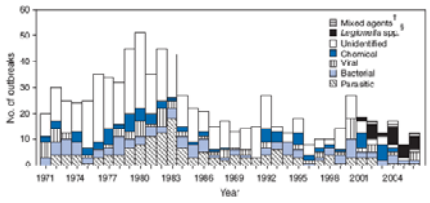
Human Enteric Viruses

Genus	Popular Name/Species	Disease caused
Enterovirus	Human Enterovirus A (CAV2-8, 10, 12, 14,16; EV71, 76, 89, 91)	Paralysis, aseptic meningitis, encephalitis, myocarditis, fever, respiratory disease, gastroenteritis, etc.
	Human Enterovirus B (CAV9, CBV1-6; E1-7,9, 11-21, 24-27,29-33; EV69,EV73-75, EV77-8, EV79-88, EV100-101	
	Human Enterovirus C CAV1, 11, 13, 17, 19-22, PV1-3	
	Human Enterovirus D EV68, 70	
Hepatovirus	Hepatitis A	Hepatitis
Reovirus	Human reovirus	Unknown
Rotavirus	Human rotavirus	Gastroenteritis
Mastadenovirus	Human adenovirus	Gastroenteritis, respiratory disease, conjunctivitis
Norovirus	Noroviruses	Gastroenteritis
Astrovirus	Human astrovirus	Gastroenteritis
Coronavirus	Human coronavirus	Gastroenteritis, respiratory disease

Bosch,1998. Int. Microbiol. 1:191-196, and Khetsuriani et al., 2006. MMWR Surveill. Summ. 55(8):1-20

Why should we be concerned about viruses in water?

Number of Waterborne Disease Outbreaks Associated with Drinking Water, by Year and Etiologic Agent — United States, 1971–2006

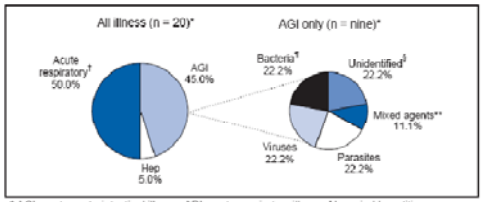


Total 814 outbreaks and 575,819 cases of illness were reported

Overall, 8% of outbreaks were caused by viruses

Yoder et al., 2008. MMWR Surveill. Summ., CDC. 57(9):39-62

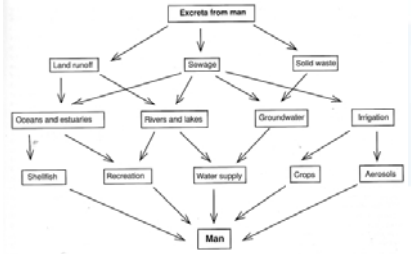
Percentage of Waterborne-disease Outbreaks Associated with Drinking water, by Illness and Etiology – United States, 2005 - 2006



* AGI: acute gastrointestinal illness; ARI: acute respiratory illness; Hep: viral hepatitis.
† All acute respiratory illness was attributed to *Legionella* spp.
§ Norovirus suspected based upon incubation period, symptoms, and duration of illness.
¶ Including one outbreak that involved multiple bacterial agents.
** One outbreak that involved bacterial and viral agents.

Yoder et al., 2008. MMWR Surveill. Summ., CDC. 57(9):39-62

Routes of Enteric Viruses Transmission

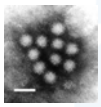


Bosch,1998. Int. Microbiol. 1:191-196

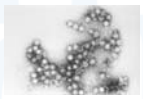
Drinking Water Contaminant Candidate List 2 (CCL 2)

- Caliciviruses
- Coxsackieviruses
- Echoviruses
- Adenoviruses

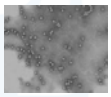
http://www.epa.gov/OGWDW/ccl/ccl2.html



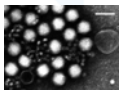
Norwalk virus
Photo credit: P. F. Williams, USEPA



Coxsackievirus B4
http://images.google.com/images?hl=en&q=Coxsackie%20virus&um=1&ie=UTF-8&sa=N&tab=wi



Echovirus
http://www.mrcvu.gla.ac.uk/research/bhelad/imagearchive1.htm



Adenovirus
Photo credit: P. F. Williams, USEPA

How do we detect viruses in water?

Virus Methods in General

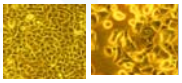
- Sample collection
- Elution
  - Beef Extract
- Reconcentration
  - Organic Flocculation
  - Celite Concentration
  - Polyethylene Glycol
- Virus assay by cell culture or molecular methods (RT-PCR, qRT-PCR)

200 – 1500 L of Water

1L

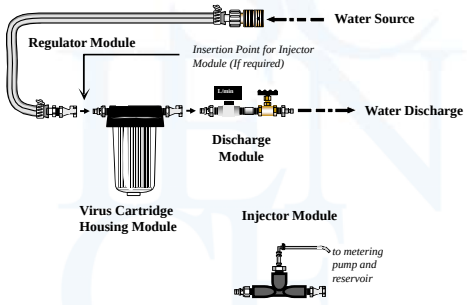
Sample concentrate (15 - 40 ml)

Cell culture assay

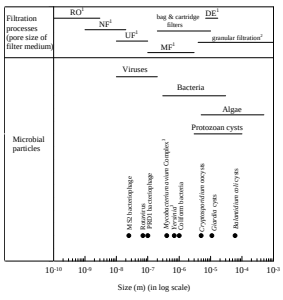


RT- PCR/ qRT- PCR

Virus Methods: sample collection apparatus



Pore Size of Filter Medium and Size of Microbial Particles



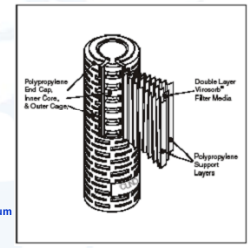
[1]RO: reverse osmosis; NF: nanofiltration; UF: ultrafiltration; MF: microfiltration; DE: diatomaceous earth filtration.
[2]Including slow sand filtration. Slow sand filter has a lower pore size than rapid-rate filter.
[3]These bacteria are rod-shape. The sizes shown represent the smallest dimension.

Types of Filters Commonly Used in Virus Concentration Procedures

- Negatively charge Filters
  - Requires conditioning the water prior to filtration
  - pH adjustment to 3.5
  - Addition of multivalent cations
- Positively charged filters
  - 1MDS electropositive filters (Cuno, Meriden, CT) are commonly used for environmental water sampling
  - Does not require conditioning the water.
  - However, requires pH adjustment for waters with pH values exceeding 8.0

Research need for virus sample collection

Virosorb® 1MDS Filter



- Recommended by ICR Method
- Charge-modified, glass and cellulose medium
- Pore size 5-8 micron
- Available in 25.4 cm cartridge
- Cost >$200

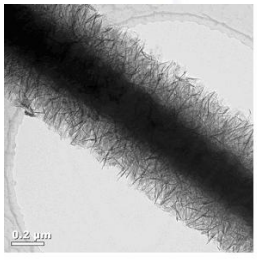
These filters are not cost-effective for routine viral monitoring

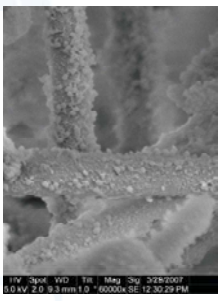
NanoCeram® Filter Characteristics

NanoCeram Biological Sampler (5")

- The active ingredient of the filter media is nano alumina (AlOOH) fiber.
- The fibers are only 2 nanometers in diameter, and 0.3 μm long and have a surface area of 500-600 m^2/g.
- The nano fibers contains naturally occurring electropositive charge.
- In NanoCeram cartridge filters, these fibers are dispersed throughout a microglass fiber matrix resulting in a non-woven media
- Size: 12.7 cm X 6.35 cm ; total surface area 316 cm^2
- Average pore size is 2 micron
- Cost approximately $40

NanoCeram® Filter





Source:http://www.argonide.com/Paper%20PREP%2007-final.pdf

Virus Sample Collection

200 – 1500 L of Water

1L

Retention of Poliovirus 1 by NanoCeram® Filters

No. of replication	Seed titer[a] (PFU)	Titer in the filtrate (PFU)	Virus Retention (%)
1	5.1×10^5	5.0×10^4	90
2	9.4×10^5	1.1×10^5	88
3	5.4×10^5	8.0×10^4	85
4	7.7×10^5	6.0×10^4	92
5	7.6×10^5	1.8×10^5	76
6	3.7×10^5	9.0×10^4	76
7	2.4×10^5	7.0×10^4	71
8	5.0×10^5	1.0×10^5	80
9	4.0×10^5	<DL[b]	100
10	6.0×10^5	1.0×10^5	83
Mean			84 (±9)[c]
Range			71-100

Poliovirus was seeded in 100 L of deionized water and filtered through NanoCeram® filters

[a]Total virus PFU in 100 liters of deionized water
[b] Detection limit
[c]Number in the parenthesis indicates standard deviation.

Virus methods: elution

Elution Scheme

- Single elution[a]
- Double elution with 1 min contact times[b]
- Double elution with 1 min, then overnight, contact times[c]
- Triple elution[d]

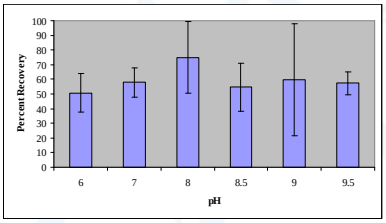
[a] **Sobsey, M. D. and A. R. Hickey. 1985.** Appl. Environ. Microbiol. **49:**259-264.
[b] **USEPA ICR method**
[c] **Dahling, D. R.** 2002. Water Environ. Res. **74:**564-568.
[d] **Dahling, D. R., and B. A. Wright.** 1984. Appl. Environ. Microbiol. **47:**1272- 1276.

Poliovirus Recovery by NanoCeram® Filters Using Six Different Elution Procedures.

Method	Elutions	Percent of virus recovery			
		Trial 1	Trial 2	Trial 3	Mean
1	1st elution	50	38	35	41 (± 8)
	2nd elution for 1 min	8	36	18	21 (±14)
	Combined percent recovery	58	74	53	62 (±11)
2	1st elution	53	62	31	48 (±16)
	2nd elution for 15 min	9	32	44	28 (±17)
	Combined percent recovery	62	93	74	77 (±16)
3	1st elution	57	60	32	50 (±15)
	2nd elution for 30 min	11	33	25	23 (±11)
	Combined percent recovery	68	93	58	73 (±18)
4	1st elution	42	57	47	48 (± 8)
	2nd elution for 60 min	10	31	24	21 (±11)
	Combined percent recovery	52	88	71	70 (±18)
5	1st elution	35	41	41	39 (± 3)
	2nd elution for 120 min	10	23	28	21 (± 9)
	Combined percent recovery	45	64	70	60 (±13)
6	1st elution	33	27	37	32 (± 5)
	2nd elution after overnight contact	9	13	10	11 (± 2)
	Combined percent recovery	42	40	47	43 (±4)

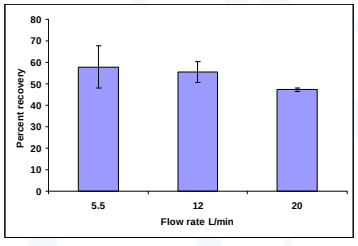
Poliovirus was seeded in 100 L of deionized water and filtered through NanoCeram® filters

Poliovirus Recovery at Different pH of Water



P=0.36

Poliovirus Recovery at Different Flow Rate



Water pH = 7; P=0.08

Recovery of Poliovirus 1, Coxsackievirus B5, and Echovirus 7 From Tap Water Using NanoCeram® Filter

Virus	Elutions	Mean percent recovery
Poliovirus 1	1st elution	35 (± 9)
	2nd elution for 15 min	19 (± 5)
	Combined percent recovery	54 (± 8)
Coxsackie B5	1st elution	18 (± 12)
	2nd elution for 15 min	9 (± 6)
	Combined percent recovery	27 (± 17)
Echovirus 7	1st elution	14 (± 6)
	2nd elution for 15 min	18 (± 9)
	Combined percent recovery	32 ± 8)

Ohio River Water Characteristics

Event	pH (range)	Turbidity (range) NTU
During 100 liter spiking experiments	7.7 (7.6-7.8)	41 (26-90)
During 10 liter spiking experiments	7.7 (7.6-7.8)	1.2 (0.17-2.75)

N=6

Comparison of Poliovirus Recovery by NanoCeram® and 1MDS Filters From Seeded Tap and River Water

Type of filter	Elution	Mean virus recovery (%)			
		100 L sample		10 L sample	
		Tap water	River water	Tap water	River water
Nanoceram®	1st elution	23 ±14	21 ±18	182 ±42	30 ±16
	2nd elution	28 ±13	16 ±15	95 ±64	25 ±15
	Combined percent recovery	51 ±26	38 ±35	277 ±22	65 ±22
1MDS	1st elution	39 ±4	25 ±20	31 ±14	13 ±4
	2nd elution	28 ±6	11 ±4	13 ±13	17 ±9
	Combined percent recovery	67±6	36 ±21	44±9	30 ±11

For 100 L samples p=>0.05; For 10 L samples, tap water p=<0.001, river water p=0.015

Comparison of Norovirus Recovery by NanoCeram® and 1MDS Filters From Seeded Tap and River Water

Type of filter	Mean virus recovery (%)	
	Tap water	River water
NanoCeram®	3.6 ± 0.6	12.2 ±16.3
1MDS	1.2 ± 1.4	0.4 ± 1.8

Norwalk virus was seeded in 10 L of dechlorinated tap water or river water and filtered through NanoCeram® or 1MDS filters

Comparison of RT-PCR Reaction Inhibition For Norwalk Virus and Poliovirus in NanoCeram® and IMDS Filters Concentrates

Type of filter	Elution	RT-PCR inhibition for norwalk virus/poliovirus					
		Tap water			River water		
		Trial 1	Trial 2	Trial 3	Trial 1	Trial 2	Trial 3
Nanoceram®	1st elution	+/ND	+/ND	+/ND	+/+	+/+	+/+
	2nd elution	+/+	+/+	+/+	+/+	+/+	+/+
1MDS	1st elution	+/ND	+/ND	+/+	+/+	+/+	+/+
	2nd elution	+/+	+/+	+/+	+/+	+/+	+/+

ND = not done
'+' indicates spiked samples were not inhibitory for RT-PCR reactions

RT-PCR Detection of Poliovirus From Seeded Tap and River Water Samples

Type of filter	Elution[a]	RT-PCR detection of poliovirus in 100 L seeded water samples[b]					
		Tap water			River Water		
		Trial 1	Trial 2	Trial 3	Trial 1	Trial 2	Trial 3
Nanoceram®	1st elution	+	-	+	-	+	+
	2nd elution	+	+	+	+	+	+
1MDS	1st elution	-	+	+	-	-	-
	2nd elution	-	+	+	+	+	-

[a]First elution was done for one minute and 2nd elution was done for 15 minutes.

[b] "+" indicates RT-PCR positive and "-" indicates RT-PCR negative

Conclusions

- The mean retention of poliovirus by NanoCeram® filters was 84 percent.
- The highest virus recovery (77%) was obtained by immersing the filters in beef extract for 1 min during the first elution and 15 minutes during the second elution.
- The recovery efficiencies of poliovirus, coxsackie B5, and echovirus 7 were 54%, 27%, and 32%, respectively.
- There was no significant difference in poliovirus recovery at tap water pH range of 6 to 9.5
- There was no significant difference in virus recovery over a water flow rates of 5.5 L/min to 20 L/min.

Conclusions

- NanoCeram® filters were comparable or better than the 1MDS filters.
- Cost approximately one-sixth of 1MDS filter, thus can be used for routine viral monitoring of water.

Published in *Applied and Environmental Microbiology.*

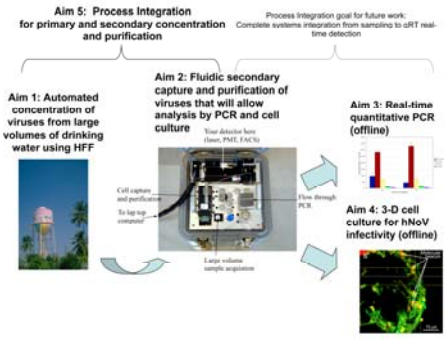
Future Directions

- This work has paved the way toward a validation project aimed at replacing the 1 MDS filter with the Nanoceram® filter in an enterovirus detection method.
- If this validation goes as expected, this new method will be considered for UCMR 3.

Automated Methods for the Quantification and Infectivity of Human Noroviruses in Water

- Timothy Straub, PI, timothy.straub@pnl.gov.
- Richard Ozanich, Co-PI, Richard.Ozanich@pnl.gov.
- Rachel Bartholomew, Co-PI, Rachel.Bartholomew@pnl.gov.
- Cindy Bruckner-Lea, Co-PI, Cindy.Bruckner-Lea@pnl.gov

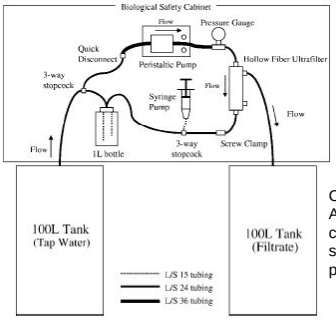
Project Overall Aims



Methods for Capturing Pathogens from Large Volumes of Water – Aim 1

- Need: Ability to efficiently capture and concentrate viruses, bacteria, and protozoa from large volumes of water
  - Pathogen concentrations in water are often very low (<1/100 mL for bacteria to <1/1,000 L for viruses)
- Methods we are investigating are mostly off the shelf technology
  - Hollow fiber filtration: Large volumes require large columns, high flow rates can be problematic
  - Sodocalcic glass wool: Very cheap, and may have great potential for viruses – investigating this summer DOE FaST team
- Modified system (next slide) may allow flow rates up to 15 L per minute

Large Scale System, Adapted from Vince Hill



Challenge: Automate to deliver concentrated samples for further processing

Secondary Concentration: The Major Bottleneck – Aim 2

- For water we get to a primary filtrate and then:
  - Centrifugation will concentrate bacteria and protozoa, but it is a manual process.
  - Viruses are left in the supernatant and still need to be concentrated.
  - Or we use single-plex immunomagnetic separation: e.g. the "disease of the day" approach, and we lose information about other pathogens.

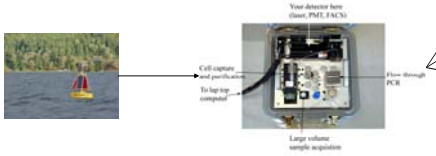
5

BEADS: Bridging the Gap between Large Volume Concentration and Detection

Sample: Large volume, matrix high, pathogens low)

Detector: Clean, tiny volume (µL-mL)

BEADS = Biodetection enabling analyte delivery system
For secondary concentration and purification



Biochip

Culture

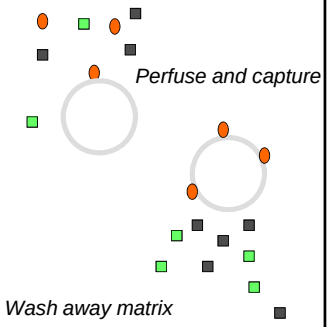
ImmunoDX

6

Biodetection Enabling Analyte Delivery System (BEADS)

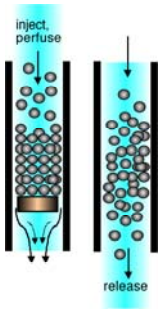
Guiding principles

1. Analytical separations can be performed on an interactive surface like a derivatized bead
2. Analytes of interest (cells, DNA, proteins, etc) are perfused over a column of beads and captured
3. Matrix materials are washed away, leaving purified analytes



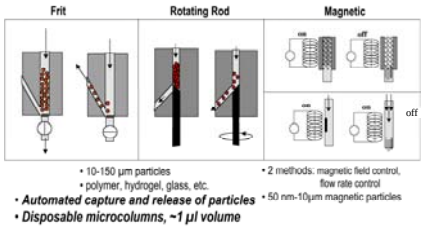
General Approach: Renewable Surfaces

- Interactive surface on beads is delivered fresh for each sample
- Compatible with users' pathogen detection requirements
  - Nucleic acid techniques
  - Cell culture
  - Immunodiagnostic "sandwich assays"
- Operates within a scaleable fluidics architecture
  - From µL to 10 L volumes
  - Architecture allows us to handle samples that are high in particulate matter and/or soluble inhibitors



Beads are flushed to waste or sent downstream for further analysis

Renewable Separation Columns (RSC) used in BEADS



Renewable separation columns are the defining feature of the BEADS platform. Depending on the user's needs, any one of these columns, and any type of separation media can be used.

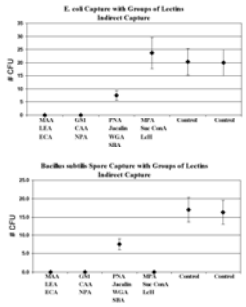
Parallel Research Tracks Include Automation and Reagent Development

- BEADS Scale up
  - Need to process large volumes (1 -10 L or greater) From a primary concentrate.
  - Baseline experiments need to show that we can capture and release pathogens as efficiently as our small systems.
  - Possible secondary concentration issues to achieve overall 10^4-10^5 concentration factor.

- Multi-agent capture
  - Default: Use multiplexed preparations of commercially (and custom) available IMS antibodies
  - New generation methods: broad spectrum capture reagents for protozoa, bacteria, and viruses.
  - How will either cell capture approach fare when challenged with low target organisms and high background flora?

Batch Trials with Lectins: Reagent Development for BEADS

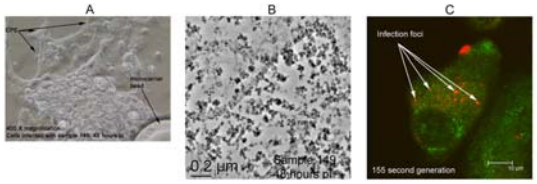
- Combinations of biotin labeled lectins were first mixed with bacteria, and then captured on streptavidin magnetic beads (indirect capture)
  - Loss of CFU indicates better capture results
  - Demonstrated capture of vegetative cells and spores.
- Challenge: direct capture.
  - Lectins conjugated to the beads do not work as well.
- Viral capture has not shown as much promise



Reverse transcription real-time PCR – Aim 3

- For human noroviruses, there is not much choice for the development of better primers and probes
  - Variations within the ORF1-ORF2 junction – most conserved to detect the most known strains.
- "Fast" vs. Slow real-time PCR
  - Newer real-time platforms allow PCR to be completed within 40 minutes. HOWEVER
  - Still need to perform reverse transcription, and that is still relatively slow
  - Your assay must be optimized for this platform…ORF1-ORF2 is not a good place to do this (secondary structure).
- For the purposes of this project, we are using the standard thermal cycling conditions.

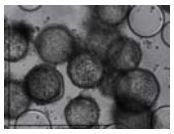
Infectivity Assays for Human Noroviruses – Aim 4

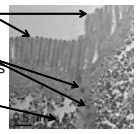


Our original work investigated the INT407 cell line grown as 3-D cell cultures. While we see evidence of infectivity, we are not observing significant viral replication. Investigations with 3-D Caco-2 cells has revealed interesting results

Observational differences between Uninfected and hNoV Infected 3-D Caco-2 cells

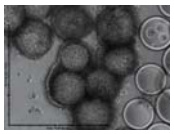
Phase contrast 4-1

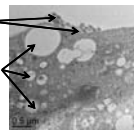




Cells challenged with hNoV negative stool samples (4-1 patient sample)

Phase contrast 4-3





Cells challenged with hNoV positive stool samples (4-3 patient sample)

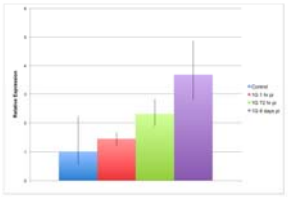
Only by TEM were differences between uninfected and infected cells easier to visualize

Real-time PCR observations indicate viral RNA replication in Caco-2 and INT407 Cells

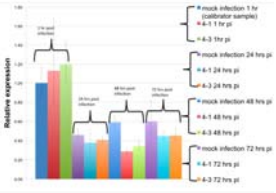
Cell line	Virus sample	Predicted copies applied to cells	Observed copies in cells 1 hr pi	Observed copies in cells 48 hrs pi	Observed copies in cells 72 hrs pi	Observed copies in cells 1 week pi
Caco-2	1G (GII)	529 ± 59	29 ± 17	11 (No sd)	2,324 ± 180	1563 ± 329
	386 (GII)	41 ± 7	Not detected	Not detected	9,375 ± 1048	Not detected
	4-3 (GI)	6,390 ± 681	171 ± 85	36,206 ± 6,244	132,919 ± 37,863	Not done
INT407	1G (GII)	529 ± 59	493 ± 28	Not done	5,370 ± 992	4,800 ± 316
	386 (GII)	41 ± 7	30 ± 51 (1/3 detect)	88 ± 77 (2/3 detect)	74 ± 126 (1/3 detect)	429 ± 363 (3/3 detect)

Possible investigation of the role of STAT-1 in controlling viral replication

STAT-1 Expression in hNoV Infected 3-D INT 407 cells



STAT-1 Expression in hNoV Infected 3-D Caco-2 cells



RNAi experiments targeting suppression of STAT-1 may help us understand its role in hNoV replication in human cells and may confirm findings about its role in limiting disease in the murine NoV model.

Research Summary

- Fluidic architecture is currently being constructed to process large volumes of water.
- Secondary capture reagents being investigated at the bench
  - Testing this summer: DOE Faculty and Student Team (FaST) will allow us test both the large volume systems and perform batch capture experiments for secondary concentration – No charge to EPA STAR
- Further investigation of Caco-2 cell line for hNoV infectivity.
  - Results have been very promising, and if there is an underlying genetic mechanism inhibiting viral replication, this could provide new insights to develop better infectivity assays.

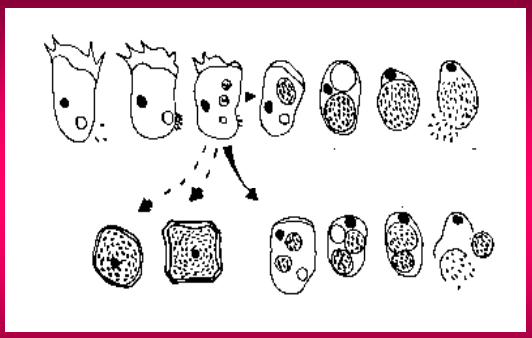
Acknowledgements

A portion of this research was performed using EMSL, a national scientific user facility sponsored by the Department of Energy's Office of Biological and Environmental Research located at Pacific Northwest National Laboratory. Funding for this work is provided by the United States Environmental Protection Agency STAR Grant Program (Grant # R833831010). The norovirus infectivity assay is jointly provided by NIAID under the Food and Waterborne Integrated Research Network Program (Contract number NO1-AI-30055) and the STAR Grant Program

This research is funded by U.S. EPA - Science To Achieve Results (STAR) Program Grant # R833831010

Food and Waterborne Diseases Integrated Research Network

Amoebae Harbor Novel Pathogens That Slip "Under the Radar Screen"



Modified from T. Rowbotham, 1986.

Recent EPA Study

Examined 40 natural water samples: (lakes, rivers, ponds, wetlands, etc.)

Examined 40 cooling tower samples

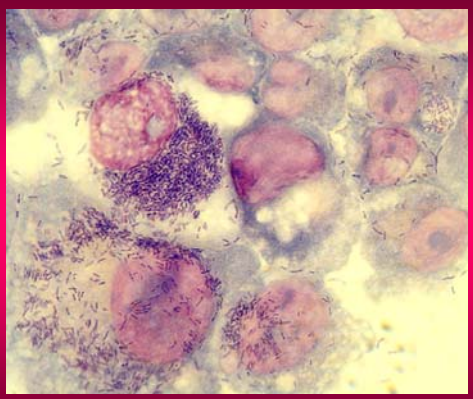
Also examined 20 other industrial: chillers, hot tubs, hot water taps/tanks, etc.

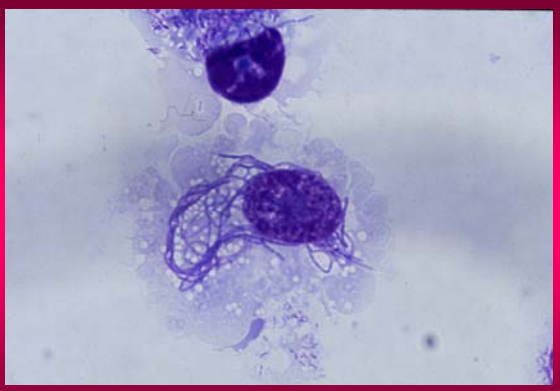
Designed a protocol to screen for infected amoebae

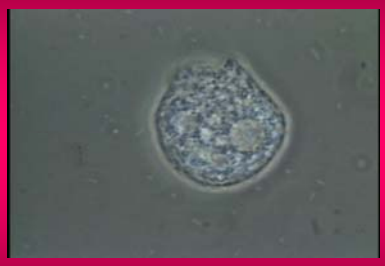
Environmental Parameters

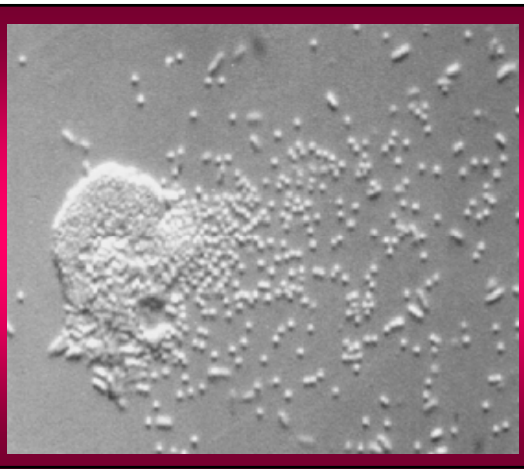
Temperature, pH, dissolved organic carbon (DOC), total nitrogen (N) and total bacteria per ml

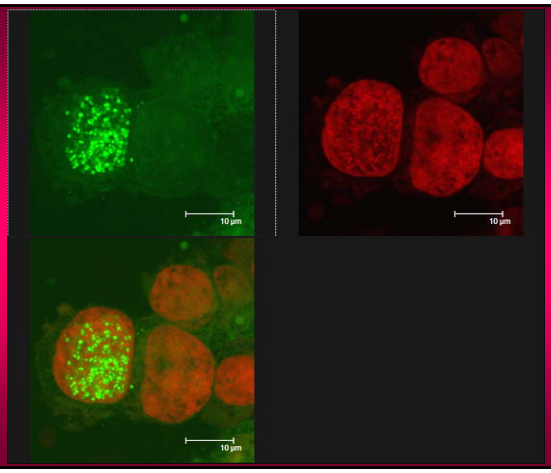
Logistic regression analyses were performed to find any parameter or set of parameters that were good predictors of the occurrence of infected amoebae



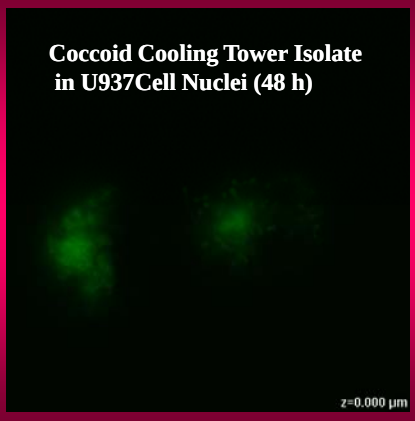


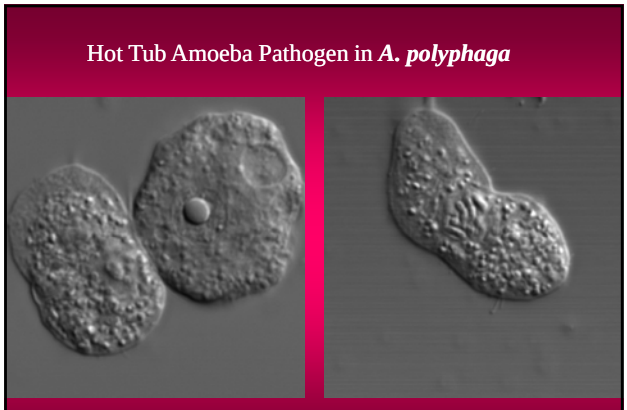




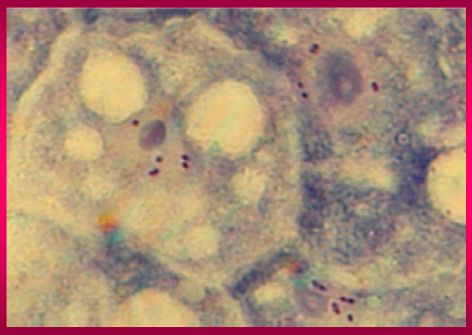



Coccoid Cooling Tower Isolate
In HeLa Cell Nuclei (48h)
z=0.000 µm

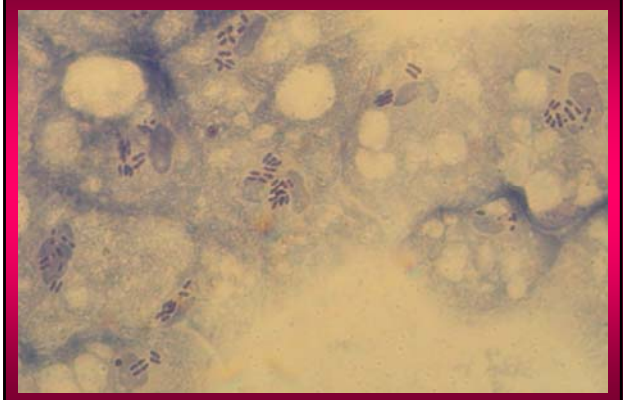

Coccoid Cooling Tower Isolate
in U937Cell Nuclei (48 h)
z=0.000 µm


Hot Tub Amoeba Pathogen in A. polyphaga

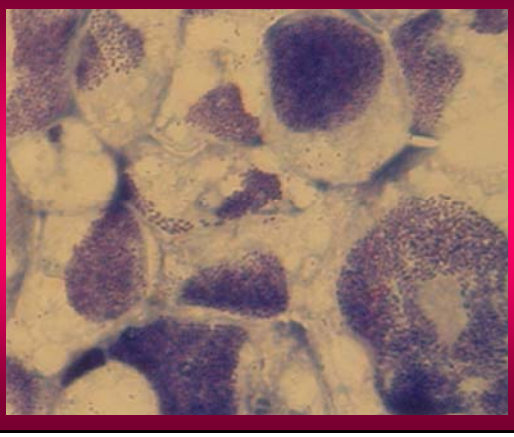
"Hot tub" infection of A. polyphaga 18 h



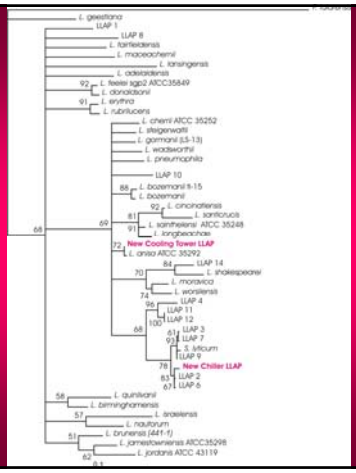
"Hot tub" infection of A. polyphaga 24 h



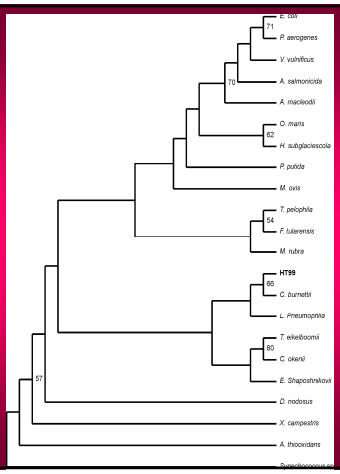
"Hot tub" infection of A. polyphaga 48 h



Phylogenetic Tree with Novel LLAPs



Phylogenetic tree with "Hot tub"



	Percent Identity					
	1	**2**	**3**	**4**		
1		94.4	86.6	89.5	**1**	CC99
2	5.8		86.3	90.2	**2**	HT99
3	13.8	14.2		85.9	**3**	*L. pneumophila* X73402
4	11.1	10.5	14.3		**4**	*C. burnetii* AY342037
	1	**2**	**3**	**4**		
	Percent Divergence					

Results

22 of 40 cooling tower samples were positive

3 of 40 natural samples were positive

2 of 20 other industrial samples were positive (hot tubs)

Odds ratio of finding infected amoebae in cooling towers vs natural environments is 16, i.e., 16 times more likely to find them in CTs (based on the way we look for them)

5 novel strains were identified, related to ***Legionella***

Only 2 of the 22 infections were from ***L. pneumophila***
And 1 of the hot tub infections was from ***L. pneumophila***

Several have not yet been isolated or identified

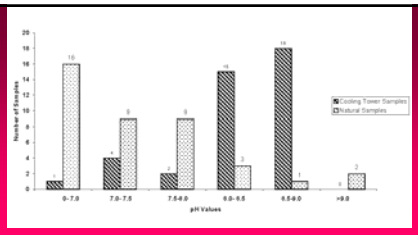
Of those that are culturable, at least 3 tested so far appear to infect human macrophages
Two non-culturable strains also infect macrophages

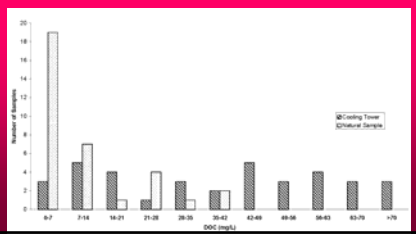
No environmental parameter was a significant predictor of occurrence of infected amoebae when cooling tower data were used alone

When data from 90 combined samples were used, pH and DOC were significant predictors

BUT cooling towers have higher pH values than almost all natural samples, and also have a higher range of DOC

Therefore it appears to be pH and DOC, but it may be something else specific to CTs that were not measured in this study





Summary/Conclusion

Occurrence of infected amoebae was significantly higher in cooling towers than in nature (16:1 odds ratio)

Non-Legionella were more common than Legionella, and half or more of these were not culturable

7 novel sequences were found, with several yet to be sequenced

Environmental parameters?? Possibly pH and DOC

Update

Several other infected amoeba specimens have been observed in the past year—

Meat industry (3)

Eyewash station (TTU)

Fish tank in public pet store

Distribution pipes (MTSU)

Pipes 1 Video Clip

Pipes 2 Video Clip

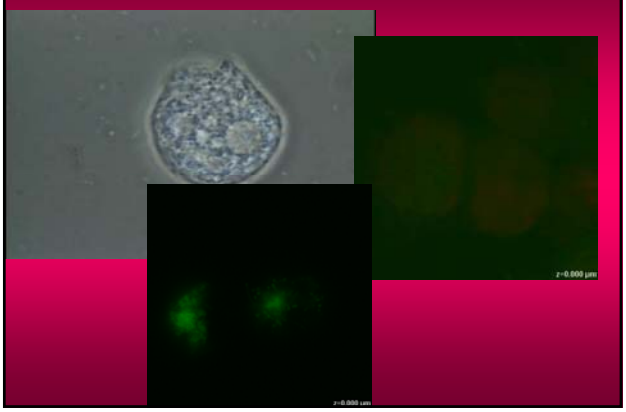
Results

Table 1. Comparison of infectivity durations of LLAPs in buffered saline vs. LLAPs in a desiccated state

Organism/Sample	Origin	Duration of Infectivity in aqueous state (days)	Duration of Infectivity in desiccated state (days)
MTM2	TN cooling tower	86	16
DSPB	water pipe	130	--
FS06*	meat industry	89	78
CSS06*	meat industry	21	78
HT7*	hot tub	130	23
LLK07*	outdoor lake	50	37
NAS03*	TX cooling tower	43	0.1
Legionella	stock culture	--	0.1

*These organisms still under study.

IF YOU LOOK, YOU WILL FIND



This research is funded by U.S. EPA - Science To Achieve Results (STAR) Program

Grant # R82711101 and R82535201

Acknowledgments

• Center for the Management, Utilization and Protection of Water Resources, Tennessee Technological Univ.
• Middle Tennessee State University

Faculty/Associates: Dr. Mary Farone, Dr. Anthony Farone, Dr. John Gunderson, Dr. Anthony Newsome, Dr. Nizam Uddin

Numerous students: Witold Skolasinski, Kate Redding, Jennifer Skimmyhorn, Elizabeth Williams, Maryam Farsian, Josh Currie, James Ventrice, Chanson Boman, Allison Reid, Marya Fisher, Jon Thomas

Detection of Various Freshwater Cyanobacterial Toxins using Ultra-Performance Liquid Chromatography-Tandem Mass Spectrometry

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Acknowledgement

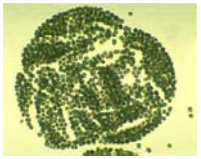
- This work was supported, in part, from the following grant
  - U.S. Environmental Protection Agency Grant (RD-83322301)
- Waters Corporation
- Lake Superior State University

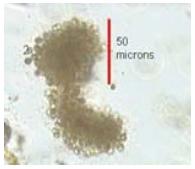
Abstract

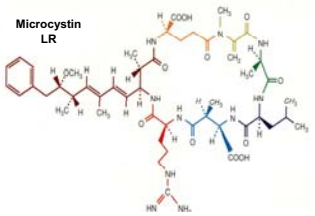
- Liquid Chromatography/Tandem-Mass Spectrometry (LC/MS/MS) is a powerful tool for the analysis of various analytes in a wide variety of matrices. What is especially attractive about LC/MS/MS is its sensitivity and selectivity. Microcystins, in particular, represent an emerging class of algal toxins of concern to the drinking water industry. Recognizing the potential health risk, the World Health Organization, Australia, and Brazil have established guidelines for the amount of microcystins permissible in drinking water (specifically microcystin LR). Recently, the United States has begun to evaluate the occurrence, health effects, and susceptibility of water treatment of algal toxins. The Environmental Protection Agency (EPA) named freshwater algal toxins to its Contaminant Candidate List (CCL).
- In this paper we investigate the use of newer technologies in smaller column packings (sub 2µm particles) to both improve the selectivity, speed, sensitivity and resolution to screen for many of these toxins (microcystins, anatoxin-a, and cylindrospermopsin) using Ultra-Performance Liquid Chromatography (UPLC®) combined with tandem mass spectrometry. Specific examples, including data from the recent Ohio river algae bloom in August, will be presented.

Sources

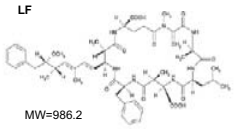
Microcystis

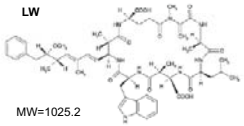


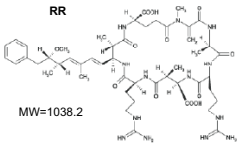


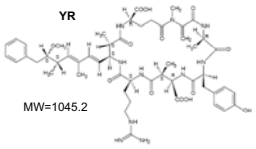


Various Microcystins



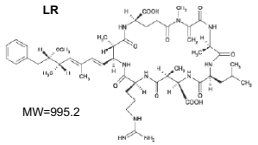






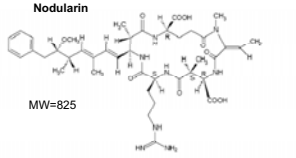
Various Microcystins and others

LR



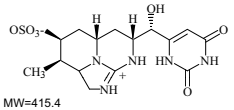
MW=995.2

Nodularin



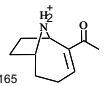
MW=825

Cylindrospermopsin



MW=415.4

Anatoxin



MW=165

Factors affecting cyanobacterial bloom formation:

- Moderate to high levels of essential inorganic nutrients (nitrogen and phosphorus)
- some are nitrogen-fixing
- water temperature 10° to 30°C
- pH levels between 6 and 9
- low flow and low turbidity
- light is not a large factor - phycobilin

Pathway/Route of Exposure

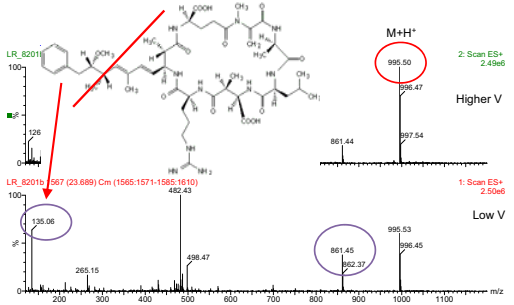
- Recreational waters - dermal, inhalation, and ingestion
- Drinking water - ingestion, dermal, ingestion.
- Dietary Supplements - ingestion
- Vegetables and Fruits - ingestion

This is a "Global Challenge"

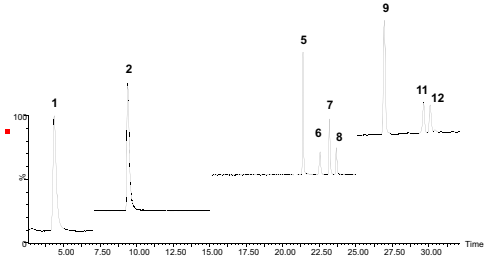
Microcystin Detection Assay

- Enzyme-linked Immunosorbent Assay (ELISA)
  - Uses polyclonal antibodies against different microcystin variants.
  - Samples are read spectrophotometrically to determine microcystin concentration.
  - Detection limit in low ppb
  - Cloudy or Murky samples pose a challenge
- High-Performance LC
  - Powerful separation capability
  - UV detection (not sensitive w/o SPE)
- LC and Mass Spectrometry
  - Offers specificity and sensitivity

Microcystin LR Mass Spectrum



Final Separation using Traditional HPLC/MS/MS

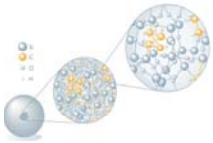


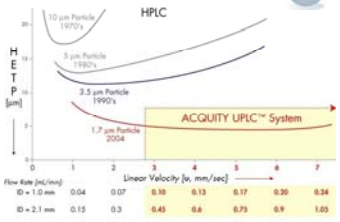
1=Cylindrospermopsin, **2**=Anatoxin-a, **5**= Microcystin RR, **6**=Nodularin, **7**=Microcystin YR, **8**=Microcystin LR, **9**=Microcystin LA, **11**=Microcystin LW, and **12**=Microcystin LF

2.1X150mm Atlantis dC_{18} (3.5µm)@30°C-0.29mL/min

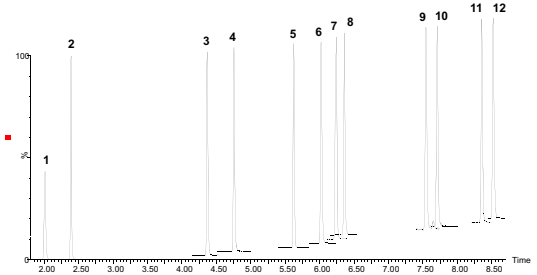
Going the Next Step...UltraPerfomance LC/MS/MS

- Small Particle (sub 2µm)
- Higher separation power
- Higher tensile strength





UPLC™/MS/MS Separation 8.5 minutes



1=Cylindrospermopsin, **2**=Anatoxin-a, **3**=Cyclo (Arg-Ala-Asp-D-Phe-Val) (IStd), **4**=[Leu^5]-Enkephalin (IStd), **5**= Microcystin RR, **6**=Nodularin, **7**=Microcystin YR, **8**=Microcystin LR, **9**=Microcystin LA, **10**=Microcystin LY, **11**=Microcystin LW, and **12**=Microcystin LF

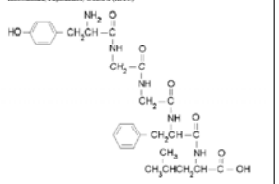
Possible Internal Standards

Peak #3

Molecular Formula: $C_{28}H_{37}N_5O_7$

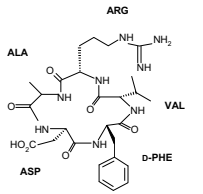
Molecular Weight: 555.62

61885 [Leu^5]-Enkephalin

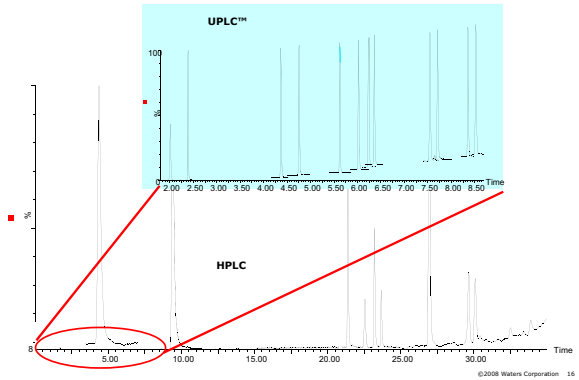


Peak #4

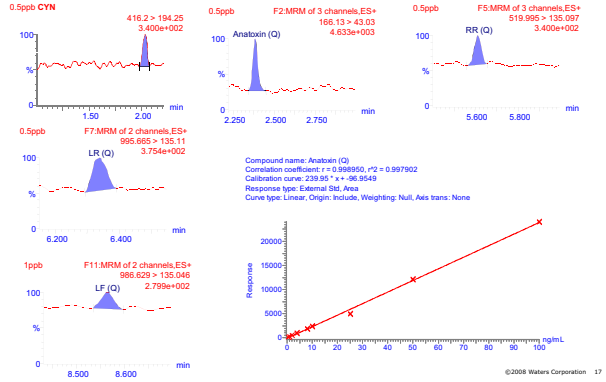
PCA-3618-PI cyclo (Arg-Ala-Asp-D-Phe-Val) (MW 588.67) $C_{27}H_{40}N_8O_8$



HPLC vs UPLC™

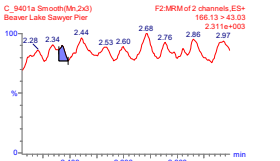


Detection Limits

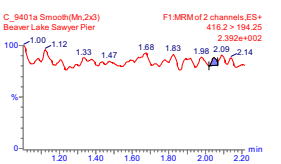


Water Samples—Filter Only (spiked at 4ppb)

Anatoxin-a

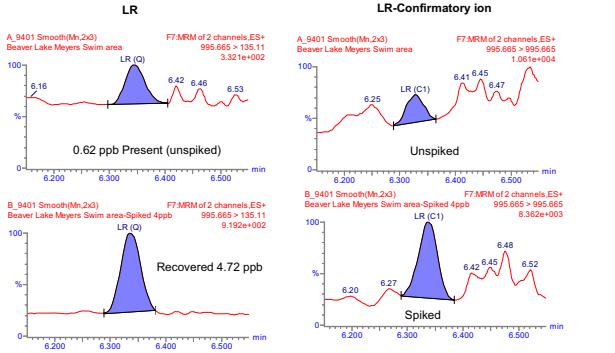


Cylindrospermopsin



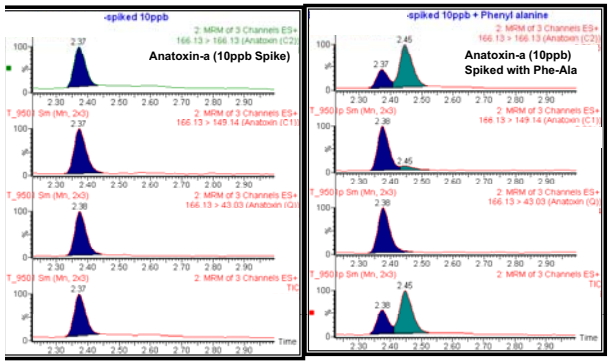
Water Samples—Filter Only (spiked at 4ppb)

Waters
THE SCIENCE OF WHAT'S POSSIBLE.



Interesting Peak in Some Water Samples at mass of anatoxin-a

Waters
THE SCIENCE OF WHAT'S POSSIBLE.



Why MS/MS is useful

Waters
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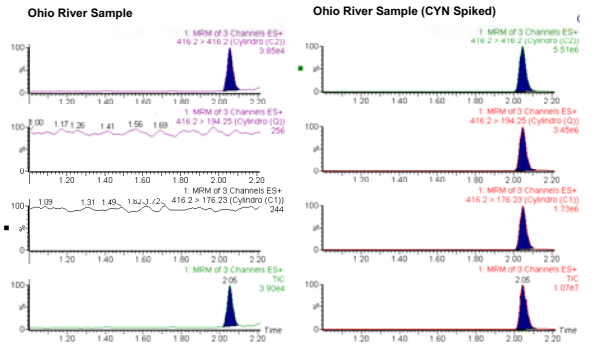

Extreme Water Sample

Waters
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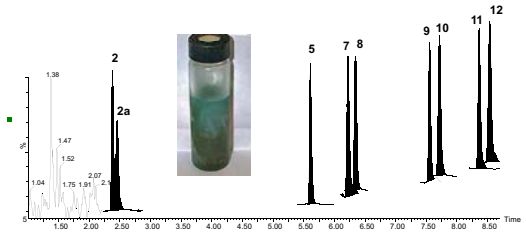
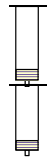


2=Anatoxin-a, **2a**=Phe-Ala, **5**= Microcystin RR, **7**=Microcystin YR, **8**=Microcystin LR, **9**=Microcystin LA, **10**=Microcystin LY, **11**=Microcystin LW, and **12**=Microcystin LF

Solid Phase Extraction (SPE) for Water Samples

Waters
THE SCIENCE OF WHAT'S POSSIBLE.

- Current methodology exist for common microcystins using C18 based SPE
- Anatoxin and cylindrospermopsin add challenges to existing SPE protocols
- VERY Preliminary work has begun on using a multimodal SPE protocol* (2 multimodal cartridges for different analytes from a single water sample)
  - Load water onto 2 cartridges in series, than separate and process each separately for the different analyte sets
  - Run 2 injections per sample (one for Cylindro, the other for anatoxin and microcystins)



*Patent applied for

Preliminary Data
Lake Water-SPE (Cylindrospermopsin)

Waters
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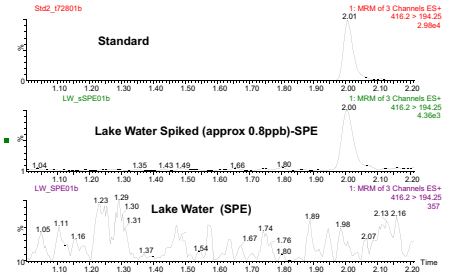
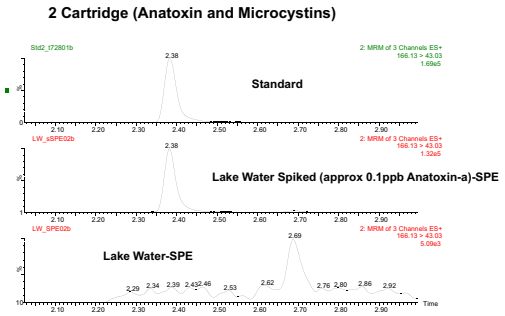
1 Cartridge (CYN only)



Lake Water-SPE (Anatoxin-a)



Conclusions

- Separation of all main Microcystins, Anatoxin-a, and cylindrospermopsin is possible in under 10 minutes using UPLC as the separation device (versus 40 Minutes by HPLC)
- MS/MS offers enhanced selectivity and sensitivity
- Combined with new SPE method, one can easily go to sub ppb levels

Acknowledgement

- This work was supported, in part, from the following grant
  - U.S. Environmental Protection Agency Grant (RD-83322301)
- Waters Corporation
- Lake Superior State University

Thank You for Attending

Questions?